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THE NUTRITION OF THE COTTON RAT (SIGMONDON HISPIDUS HISPIDUS)[1]

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ONE FIGURE

(Received for publication August 2, 1943)

Armstrong ('39) has reported that poliomyelitis can be transmitted to the cotton rat and Jungeblut ('40) found that these animals were susceptible to diphtheria infection and intoxication. Clark and Jungeblut ('40) concluded that the cotton rat does not require vitamin C. The apparent increasing importance of this animal for experimental work seemed therefore to justify a further study of its nutritional requirements.

Twelve pairs of animals were obtained from Dr. B. J. Meyer of the Zoology department in June 1942. They have been maintained on the Steenbock stock ration no. 15 and have produced approximately 400 offspring in 1 year. Meyer ('42) has made extensive studies on the breeding conditions, the reproduction and life cycle as well as the endocrine systems of the cotton rat. The young are born with a coat of fur; their eyes open in 24 hours, and they can be weaned at the age of 10 days. We have found, however, that animals weaned at the age of

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the National Live Stock and Meat Board made through the National Research Council. We are indebted to Merck and Company, Rahway, New Jersey, for supplies of thiamine, nicotinic acid, riboflavin and pyridoxine; to Abbott Laboratories, North Chicago, Illinois, for the haliver oil; and to Wilson and Company, Chicago, Illinois, for the liver extracts.

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3 weeks and weighing between 20 and 25 gm. give the most satisfactory experimental results. A growth period of 6 weeks was used for all of the studies reported in this paper. At the end of this time normal animals weighed between 60 and 70 gm., while the adult rats weighed 140–160 gm. Growth of male and female animals proceeds at approximately the same rate (fig. 1) although the female is usually slightly smaller.

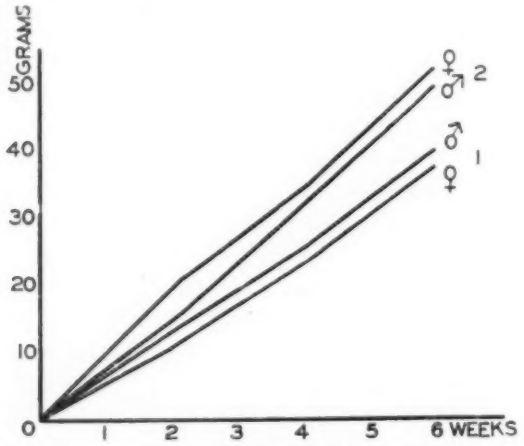

Fig. 1 Growth curves of male and female cotton rats. (1) on ration 801; (2) ration 801 plus 4% 1:20 extract.

EXPERIMENTAL

Since the cotton rat grew well and reproduced on the stock ration, animals on this ration were used as controls for further dietary studies. Experiments were first devised to determine whether the young rat could survive on the synthetic ration which supports growth and reproduction of the white rat. This ration (800) consists of casein,[2] 18%; sucrose, 73%; salts IV,[3] 4%; corn oil,[4] 5%; thiamine, 250 μg.; riboflavin, 300 μg.; nicotinic acid, 2.5 mg., pyridoxine, 250 μg.; pantothenic acid, 2 mg.; and choline, 100 mg. per 100 gm. of ration. Two drops

[2] Labco from Borden and Company, New York City.
[3] Phillips and Hart (J. Biol. Chem., vol. 109, p. 657, 1935).
[4] Mazola.

of halibut liver oil were fed bi-weekly. Three groups of six animals each were placed on the following rations: (1) stock ration; (2) ration 800 plus 2% 1:20 liver extract; (3) ration 800. The animals of the group receiving ration 800 grew poorly, averaging 3.5 gm. per week. Four of these animals showed a loss of hair on the sides of their bodies. Since Woolley ('41) reported that inositol is an antialopecia factor for mice, it was thought this factor might be required by the cotton rat.

To study the effect of adding inositol and p-aminobenzoic acid to the synthetic ration, three groups of three rats each were fed the following diets: (1) ration 800 plus 2% 1:20 liver extract; (2) ration 801 (800 plus 100 mg. of inositol and 30 mg. of p-aminobenzoic acid per 100 gm. of ration); (3) basal ration 800. The animals receiving inositol and p-aminobenzoic acid grew considerably better than those on the basal ration but poorer than those which received the liver extract. The growth rates were 8.6, 5.6 and 2.9 gm. per week respectively. The individual effects of these compounds on growth were then studied. Four groups of rats were fed the following rations: (1) ration 800; (2) 800 plus 30 mg. of p-aminobenzoic acid per 100 gm. of ration; (3) 800 plus 100 mg. of inositol per 100 gm. of ration; (4) ration 801. Rats which received inositol alone grew at approximately the same rate as those receiving ration 801 (5.8, and 5.2 gm. per week respectively), whereas those receiving the p-aminobenzoic acid alone grew at a rate of 2.9 gm. per week.

Since the cotton rat could be maintained on a synthetic diet a study of the other vitamin requirements was possible. The thiamine, riboflavin, pantothenic acid, pyridoxine, and nicotinic acid requirements have been studied as well as additional growth promoting substances found in crude materials.

Thiamine

To determine the approximate requirements of thiamine the following experiment was set up: A thiamine-free synthetic ration similar to ration 801 in respect to the other vitamins and supplemented with 2% sulphited 1:20 liver extract

was used for the basal ration. Five groups of rats were given this ration supplemented with 0, 40, 60, 80 and 150 μg. of thiamine per 100 gm. of ration. All rats on every level except the 150 μg. level became polyneuritic before the experiment terminated. The rats on the 150 μg. level grew at a rate comparable to the growth obtained on the optimum synthetic diet, 5.2 gm. per week, but the growth was less than was observed on a synthetic diet supplemented with liver extract. The same convulsive characteristics are observed with polyneuritis in cotton and white rats.

Riboflavin

For a study of the riboflavin requirements a basal diet (801) without riboflavin was supplemented with 0, 25, 50, 80 and 300 μg. of riboflavin per 100 gm. of ration and was fed to five groups of three rats each. All rats survived the experimental period and grew 1.1, 1.0, 1.7, 3.0 and 6.4 gm. per week respectively. The rats on low riboflavin diets showed no skin symptoms, but those on the suboptimal levels of riboflavin were extremely nervous and excitable, whereas those on the complete ration were more docile. Eighty micrograms of riboflavin per 100 gm. of ration is suboptimal for normal growth of the cotton rat on our synthetic diet.

Pantothenic acid

A synthetic ration which contained 0, 200, 400, 600 and 800 μg. levels of pantothenic acid per 100 gm. was fed to five groups of cotton rats. In contrast to the reports on the white and piebald rats neither greying of the fur nor adrenal hemorrhage was observed; however animals on the basal ration developed a severe dermatitis. The skin over the entire body became crusted and cracked open, causing large sores. None of these animals survived the 6 weeks experimental period. Rats which received the 200 μg. level of pantothentic acid grew poorly, whereas those on the higher levels, 400, 600 and 800 grew at approximately the same rate, 5.0, 5.0, and 5.3 gm. per week, respectively, but those which received the 800 μg.

level appeared to have a better skin and fur coat than those on the lower levels. The growth responses with the same levels of pantothenic acid are similar to those observed with the piebald rat (Henderson et al., '42).

Pyridoxine

Experiments similar to those above were devised to study the pyridoxine requirement of the cotton rat. A synthetic diet free of pyridoxine and containing only 2% corn oil was used as the basal ration. Groups of three rats each were placed on the basal ration supplemented with 0, 30, 60 and 100 μg. of pyridoxine per 100 gm. of ration. The various levels of pyridoxine gave a better growth curve than any of the other vitamins, being 2.2, 4.0, 5.0 and 6.6 gm. per week for the basal and respective levels. Symptoms other than poor growth in pyridoxine deficient animals were absent. The hair coat was excellent in most cases although the nose and mouth were slightly inflamed on some of the animals on the basal ration. However, there was no evidence of scaly skin or dermatitis.

Nicotinic acid

Cotton rats placed on a synthetic diet free of nicotinic acid for a 6-week experimental period have consistently shown poorer growth than those on a complete synthetic ration containing 2.5 mg. of nicotinic acid per 100 gm. of ration. Several of the rats on the basal ration died before completion of the experimental period. No severe mouth lesions have been noted but in some cases the mouths of cotton rats on the basal ration appeared to be slightly inflamed. Rats on the basal ration grew at an average rate of 2.9 gm. per week. Those on the complete ration grew at an average of 5.7 gm. per week. No further attempts have been made to determine the level of nicotinic acid required by the cotton rat.

Crude materials

Previous experiments indicated that synthetic diets supplemented with 1:20 liver extract produced greater growth re-

TABLE 1

Growth rates of cotton rats on synthetic rations.

	NUMBER OF RATS	GROWTH
		gm./wk.
Inositol studies		
Steenbock Stock ration	15	9
Synthetic 801 + 2% 1:20 liver extract	9	8.6
Synthetic 800 (no inositol or p-aminobenzoic acid)	14	2.9
Synthetic 800 + 30 mg. p-aminobenzoic acid/100 gm.	3	2.8
Synthetic 800 + 100 mg. inositol/100 gm.	6	5.9
Synthetic 801 (synthetic 800 + 30 mg. p-aminobenzoic acid + 100 mg. inositol/100 gm.	34	5.6
Thiamine		
Basal synthetic thiamine ration + 2% sulphited 1:20 liver extract	6	Polyneuritis 3 wks.
Basal + 40 μg. thiamine/100 gm. ration	3	Polyneuritis 4 wks.
Basal + 60 μg. thiamine/100 gm. ration	3	Polyneuritis 4 wks.
Basal + 80 μg. thiamine/100 gm. ration	3	Polyneuritis 5 wks.
Basal + 150 μg. thiamine/100 gm. ration	2	5.2
Riboflavin		
Synthetic 801 — riboflavin (riboflavin basal)	9	1.3
Basal + 25 μg. riboflavin/100 gm.	3	1.0
Basal + 50 μg. riboflavin/100 gm.	3	1.7
Basal + 80 μg. riboflavin/100 gm.	3	3.0
Synthetic 801 (300 μg. riboflavin/100 gm.)	3	6.4
Pantothenic acid		
Synthetic 801 — pantothenic acid (pantothenic acid basal)	10	died 3–6 wks.
Basal + 200 μg. pantothenic acid/100 gm.	3	2
Basal + 400 μg. pantothenic acid/100 gm.	2	5
Basal + 600 μg. pantothenic acid/100 gm.	3	5
Basal + 800 μg. pantothentic acid/100 gm.	3	5.3
Pyridoxine		
Synthetic 801 — B_6 (B_6 basal)	5	2.0
Basal + 30 μg. B_6/100 gm.	3	4.0
Basal + 60 μg. B_6/100 gm.	3	5.0
Basal + 100 μg. B_6/100 gm.	3	6.6
Nicotinic acid		
Synthetic 801 — nicotinic acid	14	2.9
Synthetic 801	13	5.7
Factors in crude products		
Synthetic 801	34	5.6
Synthetic 801 + 2% 1:20 liver ext.	6	8.6
Synthetic 801 + 4% 1:20 liver ext.	9	8.2
Synthetic 801 + 6% 1:20 liver ext.	2	5.2
Synthetic 801 + 2% sol. liver ext.	2	5.7
Synthetic 801 + 4% sol. liver ext.	2	4.1
Synthetic 801 + 5% skim milk powder	4	4.2
Synthetic 801 + 1.5 μg. biotin/10 gm.	3	4.9
Synthetic 801 + 5% grass juice powder	3	6.8

sponses than the synthetic diet alone. Three groups of rats were fed ration 801 supplemented with 2, 4, and 6% 1:20 liver extract to determine the level at which optimum growth could be obtained. The rats receiving the 2 and 4% levels grew at approximately the same rate, 8.6 and 8.2 gm. per week respectively. This was considerably better than the growth obtained on the 6% level which was approximately the same as that obtained on the synthetic alone, 5.2 gm. per week. The animals on the 6% level suffered from diarrhea which may account for the poor growth obtained on this level.

Other crude materials were fed to determine the distribution of the growth promoting substances that appear in the 1:20 liver extract. Solubilized liver extract was fed at levels of 2 and 4%. Rats on these rations failed to show any increase in growth over those which received the synthetic ration. The respective weight gains were 5.7 and 4.1 gm. per week. Synthetic diets supplemented with 5% skim milk powder produced even poorer growth than the synthetic ration alone, 4.2 gm. per week. Addition of a biotin concentrate at a level which supplied 15 μg. per 100 gm. of ration failed to produce any growth response over that obtained on ration 801 alone. On the other hand when the synthetic ration 801 was supplemented with 5% of dried grass juice a slight growth response was noted. Rats on this ration grew at a rate of 6.8 gm. per week. The average weekly gains of all animals studied are shown in table 1.

DISCUSSION

Our results with the breeding and weaning of cotton rats for experimental purposes closely parallel the work reported by Meyer and Marsh ('43) except that our animals were kept on shavings. The absence of feces in the cages indicated that considerable coprophagy takes place. Furthermore, we have not been able to obtain reproduction with animals on our stock ration when they are kept on screens. No difficulties with parasites or infection have been encountered, and no alopecia has been observed in rats on the stock ration. None of our animals are sufficiently tame to handle with ease although

several animals seem tamer than the original stock. However, the maintenance of stock animals and the production of young for experimental purposes can easily be accomplished with proper care.

The B vitamin requirements of the white rat and the cotton rat are similar in many respects, table 2. For optimum growth on the synthetic ration these animals require a minimum of 150 μg. of thiamine per 100 gm. of ration. Eighty micrograms of riboflavin were not sufficient to maintain the cotton rat, but 300 μg. per 100 gm. of ration were adequate. Pantothenic acid

TABLE 2

Vitamin B requirements of the cotton rat compared to the white rat. (μg. per 100 gm. of synthetic ration)

	COTTON RAT	WHITE RAT
Thiamine	150	80–150
Riboflavin	80 > < 300	100–150
Pyridoxine	100	80–100
Pantothenic acid	800	800
Nicotinic acid	< 2,500	Not required
Inositol	< 100,000	Not known
Choline	< 100,000	< 100,000

must be supplied at a level of 800 μg. per 100 gm. of ration to obtain optimum growth and appearance. One hundred micrograms of pyridoxine are sufficient. Preliminary work indicates that choline is also required by the cotton rat. The addition of biotin to our synthetic ration did not produce additional growth.

On the other hand, it was necessary to add nicotinic acid and inositol to the synthetic ration to obtain normal growth. The minimum requirements for these substances have not been determined. Levels of 2.5 mg. of nicotinic acid and 100 mg. of inositol per 100 gm. of the ration were used.

A further increase in growth with the addition of 1 : 20 liver extract indicates the presence of other factors which are necessary for the maximum growth of this animal. These factors which are necessary for maximum growth are not supplied by

solubilized liver which contains the biologically active substance, "folic acid". Skim milk powder also failed to show any activity, although dried grass juice powder seemed to have a limited amount of the active materials.

SUMMARY

Cotton rats require thiamine, riboflavin, pantothenic acid, pyridoxine and choline in approximately the same quantities as the white rat; in addition they require nicotinic acid and inositol. Rats on a completely synthetic diet grow at a rate of only 5.6 gm. per week.

Additional growth promoting substances are present in 1 : 20 liver extract. This is indicated by the increase in growth rate to 8.6 gm. per week when 2% liver extract is added to the ration.

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THE EFFECT OF EXCESSIVE DIETARY SODIUM AND POTASSIUM ON THE CARBOHYDRATE METABOLISM OF NORMAL RATS [1] [2]

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TWO FIGURES

[Received for publication April 29, 1943]

INTRODUCTION

A number of studies of blood sugar levels have suggested that sodium salts potentiate the action of insulin and that potassium salts have an antagonistic action toward this hormone (McQuarrie, Thompson and Anderson, '36, Adlersberg and Wachstein, '37, Silvette, Britton and Kline, '38 and Orten and Devlin, '40). Many of these reports indicate further that the action of the sodium salts is manifested to a greater degree in diabetic than in normal animals. Administered electrolytes have also been shown to affect the deposition of liver glycogen (Crabtree and Longwell, '36; Silvette, Britton and Kline, '38), a fact which suggests that the blood sugar changes may be secondary to changes in the rates of glycogenesis and glycogenolysis. That a change in the rate of oxidation of glucose may also be a factor is indicated by the reports of Johnston and Maroney ('35), Bruman and Finkelstein, ('36) and McQuarrie, Thompson and Anderson ('36).

[1] From a dissertation presented to the Graduate School of the University of Colorado by Robert C. Lewis, Jr., in partial fulfillment of the requirements for the Ph. D. degree.

[2] Presented in part before the American Physiological Society, Chicago, Illinois, in April, 1941.

The present investigation was conducted in an attempt to clarify the mode of action of the electrocytes in influencing carbohydrate metabolism in the albino rat. Glucose tolerance, the tolerance to administered insulin, glycogen storage and respiratory quotients were compared in rats which had been maintained on diets differing only in their content of sodium chloride and potassium chloride.

EXPERIMENTAL

Animals and diets

Male rats of the Yale strain which weighed about 200 gm. were maintained on a basal diet to which different salt mixtures were added. The paired feeding technique was used during a preliminary period of 2 weeks. Each group consisted of three animals: one received the unmodified control diet, another the high sodium diet and the third the high potassium diet. The basal diet consisted of starch, 450 gm., casein, 325 gm., unsalted butter, 80 gm., cod liver oil, 20 gm., dried yeast, 80 gm. and powdered agar, 20 gm. per kilogram of diet. The control diet contained 25 gm. per kilogram of a salt mixture which was essentially mixture 351 of Hubbell, Mendel and Wakeman ('37). The high sodium diet contained 20 gm. of a potassium free salt mixture and 90 gm. of extra sodium chloride per kilogram of mixed diet. The high potassium diet contained 20.5 gm. of a sodium free salt mixture and 50 gm. of extra potassium chloride.

Glucose tolerance tests

At the end of the feeding period the groups of three animals were fasted 24 hours and their tolerance was determined to either 0.25 gm. or 0.5 gm. of glucose per 100 gm. of body weight administered by stomach tube. The blood sugar analyses were done by the micro method of Somogyi ('37) on duplicate 0.05 ml. samples of blood from the tail vein.

The range and distribution of the fasting blood sugar levels were approximately the same in each dietary group. The tol-

erance of animals on the three diets to 0.25 gm. of glucose per 100 gm. of body weight showed no consistent differences. When the tolerance was determined following the administration of 0.5 of glucose per 100 gm., the animals on the high sodium diet demonstrated a tolerance which was slightly greater than that of the controls and those on the high potassium diet. Figure 1 shows the mean values for seven animals on each diet. In five of the seven groups the animal

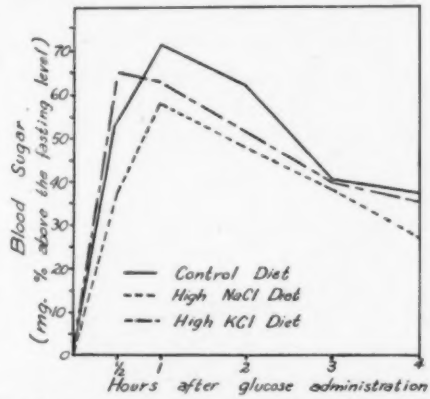

Fig. 1 Glucose tolerance curves after the oral administration of 0.5 gm. of glucose per 100 gm. of body weight. The values show the mean of the determinations on seven animals on each diet.

on the high sodium diet had a lower curve. However, statistical analysis by the *t*-test [3] reveals that only the values at the second hour after glucose administration are significantly different ($P < 0.02$). There was no significant difference in the glucose tolerance between the control animals and those fed excessive potassium.

Insulin tolerance tests [4]

The preliminary feeding period and 24-hour fast were the same as in the previous experiment. Each animal then re-

[3] Significant differences for small groups of animals were determined by "Student's" method (cf. Fisher, '34). By this method significant differences are indicated when $P = 0.05$ or less (Fisher, p. 158).

[4] The insulin used was furnished by Eli Lilly and Co., Indianapolis, Indiana. We gratefully acknowledge their cooperation.

ceived 0.3 to 0.5 units (the same within each group) of regular insulin subcutaneously per 100 gm. of body weight. Figure 2 gives the mean blood sugar values for seven groups of animals. In six of the animals fed the high sodium diet the initial fall of the blood sugar was faster than in the corresponding control. Application of the *t*-test to these values for the first half hour following the administration of insulin shows that $P < 0.05$. Values for the first, second and third hour blood sugars are not significantly different. The return

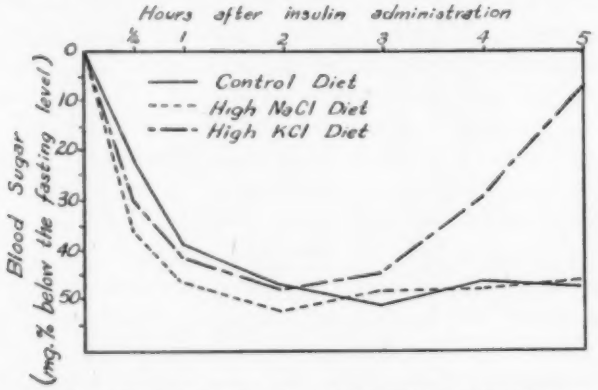

Fig. 2 Blood sugar curves after the adminstration of insulin. The values show the mean of the determinations on seven animals on each diet.

of the blood sugar toward the normal level at the 4-hour period was consistently faster in the animals which had been fed the high potassium diet than it was in the others. The difference between these animals and the controls is significant ($P < 0.05$). Insulin shock occurred in five of the animals on the high sodium diet, in three of the controls, and in only one of those on the high potassium diet.

Glycogen deposition

Nine groups of animals were maintained through a preliminary feeding period as described above. After a 24–hour fast they were given 0.25 gm. of glucose per 100 gm. of body

weight by stomach tube. Two hours later they were anesthetized with sodium amytal. The livers were removed and frozen immediately in a mixture of solid carbon dioxide and ether, and the gastrocnemius muscles were frozen in situ. These tissues were crushed to a fine powder (cf. Graeser,

TABLE 1

Determinations of glycogen and respiratory quotients.

THE VALUES FOR LIVER AND MUSCLE GLYCOGEN 2 HOURS AFTER THE ADMINISTRATION OF 0.25 GM. OF GLUCOSE PER 100 GM. BODY WEIGHT FOLLOWING A 24-HOUR FAST

DIET	NUMBER OF ANIMALS	MUSCLE GLYCOGEN GM. PER 100 GM.			LIVER GLYCOGEN GM. PER 100 GM.		
		High	*Low*	*Mean*	*High*	*Low*	*Mean*
Control	9	0.57	0.43	0.50	2.76	1.30	2.01
High NaCl	9	0.60	0.45	0.52	2.15	1.55	1.79
High KCl	9	0.61	0.42	0.52	2.47	1.21	1.59

VALUES FOR LIVER GLYCOGEN AFTER A 12-HOUR STANDARD FEEDING PERIOD FOLLOWING A 24-HOUR FAST

DIET	NUMBER OF ANIMALS	LIVER GLYCOGEN GM. PER 100 GM.		
		High	*Low*	*Mean*
Control	10	5.96	2.20	4.41
High NaCl	10	7.41	3.40	5.26

MEAN VALUES FOR RESPIRATORY QUOTIENTS AFTER THE ADMINISTRATION OF 0.25 GM. OF GLUCOSE PER 100 GM. BODY WEIGHT FOLLOWING A 24-HOUR FAST

DIET	NO. OF ANIMALS	RESPIRATORY QUOTIENTS					
		Fasting	Hours after glucose				
			0–1	1½–2½	3–4	4½–5½	7½–8½
Control	8	.732	.806	.857	.816	.766	.727
High NaCl	8	.723	.818	.775	.755	.727	..
High KCl	8	.730	.798	.829	.779	.758	.729

Ginsberg and Friedemann, '34), mixed and sampled in duplicate for analysis by the method of Good, Kramer and Somogyi ('33). The results are summarized in table 1. The amount of glycogen in the muscles was approximately the same in the three dietary groups. The storage of liver glycogen by the animals on the high sodium diet did not differ

significantly from that of the controls. However, the animals which received the high potassium diet consistently stored less glycogen than the controls. The *t*-test for significance of this difference shows that $P < 0.01$.

A study of the effect of a high sodium diet on liver glycogen storage by a different technique is also summarized in table 1. The glycogen determinations were made following the principles outlined by Guest [5] ('41). Following the preliminary feeding period the rats were fasted 24 hours. They were then given 5 gm. of the mixed diet and 12 hours were allowed for its consumption. At the end of the 12 hours the liver glycogen was determined in the manner described above. The glycogen storage of animals which had received the high sodium diet was greater than that of the controls in eight of ten pairs. The *t*-test shows that this difference is significant ($P < 0.05$).

Repetition of the studies of Crabtree and Longwell ('36), in which the liver glycogen was determined after a 24-hour fast, failed to show any significant difference between control animals and those fed excessive sodium. The individual variations in a series of twenty-two pairs were very large, and there was no consistent trend in the animals which had received the experimental diet. The mean values for experimental and control animals were 0.285 gm. and 0.338 gm. per 100 gm. of liver, respectively.

Respiratory quotient studies

Eight groups of animals were studied. At the end of the preliminary feeding period the animals were fasted for 24 hours and the gaseous exchange for a 1-hour period was determined by the method of Haldane (1892). The rats were then given 0.25 gm. of glucose per 100 gm. of body weight by stomach tube and the gaseous exchange was determined at intervals thereafter. Each determination was carried through a 1-hour period, and the recorded R. Q. represents

[5] Dr. Guest outlined his method to us in 1939, prior to the publication of his results. We are indebted to him for this information.

the total respiratory exchange for that hour. The results of this experiment are recorded in table 1. There was virtually no difference between the fasting R. Q. of the three dietary groups. The change during the first hour after the administration of glucose was of about the same magnitude in all three groups. The R. Q. of the animals which had received high sodium diets then decreased during the second hour, whereas that of the control animals and those on the high potassium diet continued to rise. The R. Q. of the former animals had returned to the fasting level within 5 hours, whereas it was still considerably above that level in animals on the other diets. Application of the *t*-test to these data shows that $P < 0.01$ for the $1\frac{1}{2}$ to $2\frac{1}{2}$-hour period, $P < 0.05$ for the 3 to 4-hour period and $P < 0.01$ for the $4\frac{1}{2}$ to $5\frac{1}{2}$-hour period. All of these differences are significant. The differences between the control animals and those on the high potassium diet were not significant.

DISCUSSION

If insulin sensitivity is judged by the initial rate of fall of blood sugar after insulin administration, by the incidence of insulin reactions, and by the rate of return of the blood sugar to normal, it can be concluded that the high sodium diet induced an increase in the sensitivity to insulin and that the diet high in potassium made the animals less sensitive. Glucose tolerance tests, on the other hand, showed only a questionable increase in the ability of the animals fed excessive sodium to utilize glucose, and they failed to show that animals fed excessive potassium had any definite change in their glucose tolerance. Inasmuch as many factors other than insulin are involved in the utilization of ingested glucose, these latter findings do not vitiate the conclusion that sensitivity to insulin was influenced by the dietary regimes employed. The choice of the Yale strain of rats for this study of "normal" animals was perhaps unfortunate in the light of the report by Cole and Harned ('38) which showed that this strain has a diabetic tendency. Their finding was confirmed

by Orten and Devlin ('40), who observed a more marked effect of sodium chloride injection on glucose tolerance in "low tolerance rats" than in "normal rats." It is possible that more significant differences in glucose tolerance might have been observed in our work if a preliminary division into low tolerance and normal animals had been made.

The liver glycogen storage following the ingestion of glucose when the preliminary diet contained excessive sodium choride was not significantly different from that of controls. However, when a 12-hour standard feeding period was used instead of the single dose of glucose, the high sodium animals stored significantly greater amounts of liver glycogen than did the controls. It should be noted that in the standard feeding period the animals received considerably more potential glucose as starch, protein and fat than did those which received a single dose of glucose. The failure to confirm the results of Crabtree and Longwell ('36) may have been due to the difference in the strain of rats used. These workers used animals from the Denver University colony. Application of the *t*-test to the individual data (unpublished) of their second series of animals shows that the differences were significant ($P < 0.01$).

Rats on a high sodium diet had a lower R. Q. following the ingestion of a measured amount of glucose than did animals on a control diet. This finding may reflect the tendency of the former to deposit more liver glycogen and thus render less glucose available for oxidation. Parallel studies of intestinal absorption and glucose excretion would probably make possible a more exact interpretation. Sayers and Orten ('41) demonstrated that there was more glycosuria when glucose in saline was administered by the intraperitoneal route than when the same amount of glucose was given in distilled water. Our procedure was not comparable to that of Sayers and Orten, but, if electrolyte feeding at this level did increase glycosuria, then our findings may have been partially conditioned by this reaction. The decreased deposition of glycogen in rats which had received a high potassium diet should

have resulted in an increase in the amount of glucose available for oxidation and, therefore, in an increase in the R. Q. when compared to that of control animals. However, the results herein reported do not indicate a significant difference in the R. Q. of the two dietary groups. It is possible that here, too, the excessive electrolyte may have induced an increased glycosuria which influenced the R. Q.

An exact explanation of the results herein reported is not immediately evident. It may be that changes in blood sugar and in the oxidation of glucose are the result of primary changes in glycogen storage, but unequivocal proof of this possibility is lacking.

SUMMARY

Male albino rats of the Yale strain were maintained for a period of 2 weeks on normal diets and on diets high in sodium or potassium. After this period determinations were made of the glucose tolerance, the response to insulin, the deposition of gycogen following the feeding of glucose or a measured amount of mixed diet, and the R. Q. in fasting animals and after the administration of glucose.

Whereas statistical analysis showed a significant difference in the values at only one point in the glucose tolerance curves, the curves of the animals fed excessive sodium tended to be lower than those of the control rats when 0.5 gm. of glucose per 100 gm. of body weight was given.

The animals which received excessive sodium were more sensitive and those which received excessive potassium were less sensitive to administered insulin than were those on a control diet.

When the liver glycogen was determined after a 24-hour fast there were no significant differences between the dietary groups. Following the administration of a measured dose of glucose the liver glycogen of the animals on excessive potassium was significantly lower than that of the control animals. Those which had received excessive sodium did not vary sig-

nificantly from the controls under these conditions. However, when a standard feeding of mixed diet was given, the results showed a significant increase in liver glycogen storage by the animals receiving excessive sodium. The dietary regimes employed had no significant effect upon the muscle glycogen.

The increase of the R. Q. following the administration of glucose was neither as marked nor as prolonged in the animals which had received excessive sodium as it was in those on a control diet. The rats which had received excessive potassium did not vary significantly from the controls.

ADDENDUM

Sayers, Sayers and Orten ('43), have recently published a report showing an increased utilization of glucose by Yale strain rats which were given sodium chloride along with glucose by intraperitoneal administration. Their results show a lower blood sugar when NaCl is given with glucose than with glucose alone. They also observed higher liver glycogen values in their NaCl treated animals. These results are in accord with our findings. They also report higher carcass glycogen in their NaCl treated animals. We did not find a significant difference in muscle glycogen values in our various groups but their findings on the whole carcass probably are more significant inasmuch as the values for one muscle do not represent a true picture of total tissue glycogen changes. These workers conclude that the effect of NaCl is to increase the storage of carbohydrate in the experimental animal, a conclusion which we have also reached by an entirely different technique.

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THE RETENTION OF THE NUTRITIVE QUALITY OF BEEF AND PORK MUSCLE PROTEINS DURING DEHYDRATION, CANNING, ROASTING, AND FRYING[1]

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The use of large quantities of meat by the armed forces and for export makes it more necessary than ever to know whether canning, dehydrating, or ordinary cooking have any damaging effect on the nutritional quality of the proteins in the meat. The researches reported on the effect of heat on meat proteins are not sufficiently harmonious to constitute a clear-cut answer to the question.

Some reports (Jarussowa, '29; Scheunert and Bischoff, '30; Scheunert and Venus, '32; Seegers, Schultz and Mattill, '36; Swanson and Nelson, '38) support the view that moist heat, such as is used in boiling and autoclaving, does not significantly lower the nutritional quality of meat proteins. On the other hand, Morgan and Kern ('34), believe that autoclaving or boiling does damage these proteins. As to the influence of dry heat, Mendel and Lewis ('14) reported that low temperature drying (55°C.) of proteins in meat had practically no effect. More severe heating (above 100°C.) causes considerable injury (Seegers and Mattill, '35). Feeding tests have shown that the drying of meat at temperatures below 80°C. gives a product whose protein is equal in nutritive quality to that of cooked meat (Bate-Smith, '42). Pro-

[1] Presented before the Division of Biological Chemistry, Detroit meeting, American Chemical Society, April 12, 1943.

teins in fish meals are apparently damaged by dry heat (Ingvaldsen, '29; Maynard and Tunison, '32; Maynard, Bender, and McCay, '32; Schneider, '32). A lowering of the digestibility appears to be largely responsible for the decreased nutritional quality of dry-heated proteins.

EXPERIMENTAL PROCEDURES

The meats studied

Raw cured pork shoulder. Fresh pork shoulders which had been boned, ground and dry-cured. After grinding the meat was mixed mechanically with the dry curing salts and then vacuumized long enough to remove air. Curing required 24 to 48 hours at 32–34°F.

Canned cured pork shoulder. A portion of the above raw cured pork which was vacuum sealed in 2½- or 6-pound tin cans and kept at 235°F. in a commercial retort until a center temperature of 225°F. was reached. The smaller cans required 3 hours whereas the larger cans required 3⅓ hours.

Roast pork shoulder. Fresh pork shoulders (butt end removed) which were roasted, two in each oven, in open trays without added water at 325° F. until an internal temperature of 185°F. had been reached, requiring 4½ to 5 hours. After removal of skin, bone, and excess fat, the meat from the shoulders was ground and mixed.

Fried pork shoulder butt. Fresh pork shoulder butts (from the shoulders above) which were sliced ½ inch thick and fried in open skillets. The slices were first quickly browned on each side at relatively high temperatures, then fried at a lower temperature for 15 minutes on each side. After removal of excess fat and bone the meat from the butts was ground and mixed.

Dehydrated pork. Fresh pork shoulders which had been boned and cut into 2-inch cubes, pre-cooked a minimum of 30 minutes after the internal temperature reached 165°F., the broth drained off, the meat ground and dehydrated to 10% moisture content in a Roto-Louvre drier at an air tempera-

ture of about 155°F. Two to 3 hours were required for drying. The broth, after removal of the fat, was concentrated and mixed with the dehydrated meat. The finished product was compressed into tin cans and sealed under vacuum.

Dehydrated beef. Fresh carcass beef which had been boned and cut into 2-inch cubes, pre-cooked a minimum of 30 minutes after the internal temperature reached 165°F., ground and finally dehydrated to 10% moisture content in a Roto-Louvre drier at an air temperature of about 155°F. Dehydration was completed in 2 to 3 hours. Finished product was packed like dehydrated pork.

In all cases the above meats were mixed into the respective diets without additional treatment other than the necessary grinding for proper mixing, and freezing for preservation until used. Dehydrated meats were reconstituted with water before mixing into the diets when comparisons were made with moist meats.

When feeding raw pork, the rats were examined for trichinae at the termination of the experiment to insure freedom from such infestation unless the pork samples had been frozen long enough to kill trichinae before starting the experiment (United States Department of Agriculture, '40).

All meats were analyzed for moisture, protein, and fat as a basis for designing the diets.

Six male weanling albino rats were started in each group of test animals. They were grouped with respect to both size and litter except in experiment 4 where rats (Sprague-Dawley) of unknown litter origin were used. All rats were housed in an air-conditioned room maintained at nearly constant temperature and humidity. Ad libitum feeding was used in all experiments except experiment 2 where paired feeding was employed. Individual weekly weight records were kept for growth periods of 8 to 10 weeks. Individual food consumption records were kept for those animals which were caged separately, but not for those in experiment 3 which were grouped six to a cage. A group food record was kept in this experiment.

The efficiency of protein utilization for growth, as proposed by Osborne et al., ('19), i. e., gain in weight per gram of protein eaten, was used as the criterion of protein quality.

The diets used

Usually all meat proteins were fed at two levels. At the higher levels, usually 17 to 20% of the solids, 10 to 15 % of the total calories were contributed by the protein. This may be considered as a practical level since the data of Berryman and Chatfield ('43) show that protein supplied 12.1% of the total calories in the United States Army diets during a 6-month period. Also Sherman ('41) states that ". . . . the results of recent research confirm and strengthen the generally accepted dietetic custom of allowing from 10 to 15% of the total food calories for protein under ordinary conditions."

The lower levels of protein, 8 to 11% of the solids or 7 to 9% of the total calories, were suboptimal for rat growth and, therefore, were suitable for a more critical evaluation of the proteins.

All diets were analyzed as a further check on their composition and the protein contents given are values calculated to the dry weight basis from the analytical data. In order that the protein contents given might represent the meat protein content, corrections were applied for the non-meat nitrogen contributed by yeast, liver concentrate powder, and corn starch when present.

With the exception of experiment 1-b, the meats were added to each diet by replacement of a weight of carbohydrate equal to that of the meat solids (dry solids were calculated from analytical data for each meat). Other ingredients are given on an air-dry basis except in experiment 4 where all ingredients are on a dry-weight basis.

The diets used in the respective experiments had the following composition:

Experiment 1-a. Each of the two meats, raw cured pork and canned cured pork, was used in amounts to provide 10,

20, and 29% of pork protein in the diets. The fat contents of the two comparable diets at each protein level were made equal by adding lard in the amount required to the diet having the lower fat content. It was necessary to omit the butter from the basal ration to make the diets containing 29% of pork proteins because of the high fat content of these diets.

The balance of the diets contained salt mixture, 5 (that of Phillips and Hart '35, modified by the addition of 0.9 gm. $MnSO_4 \cdot H_2O$ per kilogram); butter, 6; dried brewer's yeast, 5; fortified sardine oil (3000 A, 400 D), 1; liver concentrate powder (Wilson's 1:20), 1; and dextrose to make 100%.

The B-complex content of the diets was supplemented by giving each rat a daily aqueous supplement of the following synthetic vitamins: thiamine, riboflavin, and pyridoxine, 20 μg. each; calcium pantothenate, 100 μg.; niacin, 1 mg.; and choline chloride, 50 mg.

Experiment 1-b. These two diets were prepared with a protein distribution as follows: Raw cured pork or canned cured pork, 7.5% of the diet; wheat, 4.8; oats, 3.3, and corn, 2.1. The complete diets contained pork (wet basis), 25%; wheat, 30; oats, 20; corn, 14; salt mixture, 5; butter, 5; and fortified sardine oil, 1.

The B-complex content of these diets was not supplemented.

Experiment 2. Raw cured pork and canned cured pork were each used at levels to provide 9.7 and 18.8% protein in the diets. Lard was added to give a protein to fat ratio of 1:2 and salt mixture and calcium carbonate were adjusted to give a calculated total calcium and phosphorus content of 1.00 and 0.67% of the solids respectively. The balance of each diet consisted of dried brewer's yeast, 2; fortified sardine oil, 1; liver concentrate powder (Wilson's 1:20), 0.5; and corn starch to make 100%.

The B-complex content of each diet was supplemented as in experiment 1-a except that the choline chloride was reduced to 15 mg. and the supplement was administered in dextrin pills.

Experiment 3. The diets used here were the same as those in experiment 1-a except that unreconstituted dehydrated beef and dehydrated pork were used as sources of protein. Both beef and pork were fed at a protein level of 20% and also at levels of 10 and 11% respectively.

Tallow was added to the beef diets to give a protein to fat ratio of 1 : 2. The B-complex content of these diets was not supplemented.

Experiment 4. Meats were used to provide protein levels as follows: Canned cured pork, 8.9 and 18.3%, roast fresh pork, 9.2 and 17.7%, fried fresh pork, 9.1 and 17.4%, reconstituted dehydrated pork, 8.3 and 17.1%, reconstituted dehydrated beef, 8.3 and 18.1%. Fat was adjusted by the addition of lard to the pork diets and tallow to the beef diets to give a protein to fat ratio of 1 : 2. Salt mixture and calcium carbonate were proportioned to give calcium and phosphorus values of 1.00 and 0.67% of the solids respectively. Fortified sardine oil was included at a 1% level and corn starch was used to bring the total solids to 100%.

The natural B-complex content of the diets was supplemented by giving daily to each rat one 50 mg. protein-free yeast extract tablet (purchased from Harris Laboratories, Tuckahoe, New York). The adequacy of B-complex intake was confirmed by the daily addition of 0.8 gm. of liver concentrate powder to the food of each rat beginning with the eighth week and continuing until the end of the experiment (end of tenth week). The growth rate was not changed by the addition.

RESULTS

The results obtained are summarized in table 1. The average gains and protein intakes for the entire growth periods of 8 to 10 weeks have been tabulated. Whenever differences were observed between comparable groups, they usually became evident about the second week and increased progressively to about the eighth week. The largest differences were observed at about the eighth to tenth week.

TABLE 1

Nutritive value of meat proteins.

EXP. NO.	PROTEIN SOURCE	PROTEIN LEVEL	NUMBER OF RATS	GROWTH PERIOD	WEIGHT GAIN AVG.	PROTEIN INTAKE AVG.	GRAMS OF GAIN PER GRAM OF PROTEIN	
							Avg.	Range
		%		*weeks*	*gm.*	*gm.*		
	Raw cured pork		5		180	66	2.73	2.39–2.91
		10		8				
	Canned cured pork		5		159	60	2.65	2.40–2.79
	Raw cured pork		5		210	112	1.87	1.76–1.95
1-a		20		8				
	Canned cured pork		5		204	106	1.92	1.79–2.14
	Raw cured pork		6		213	148	1.44	1.35–1.50
		29		8				
	Canned cured pork		6		207	147	1.41	1.31–1.51
	Raw cured pork + cereals		6		207	128	1.62	1.41–1.78
1-b		17.7		8				
	Canned cured pork + cereals		6		181	117	1.55	1.35–1.69
	Raw cured pork		6		135	62	2.18	1.95–2.42
		9.7		10				
2	Canned cured pork		6		124	62	2.00	1.59–2.22
	Raw cured pork		6		191	100	1.91	1.81–2.02
		18.8		8				
	Canned cured pork		6		194	99	1.96	1.71–2.29
	Dehydrated pork	11	6		174	72	2.42	
				9				
3	Dehydrated beef	10	6		160	61	2.62	Group
	Dehydrated pork		6		237	127	1.98	caging
		20		9				
	Dehydrated beef		6		226	120	1.97	
	Canned cured pork	8.9	5		126	55	2.29	2.20–240
	Roast fresh pork	9.2	5		149	65	2.29	2.20–2.33
				10				
	Fried fresh pork	9.1	6		175	68	2.57	2.51–2.66
	Dehydrated pork	8.3	6		155	56	2.77	2.62–2.86
	Dehydrated beef	8.3	6		114	50	2.28	2.05–2.40
4	Canned cured pork	18.3	6		256	144	1.78	1.64–1.90
	Roast fresh pork	17.7	6		262	139	1.88	1.75–2.09
				10				
	Fried fresh pork	17.4	6		282	146	1.93	1.76–1.99
	Dehydrated pork	17.1	5		259	139	1.86	1.73–2.03
	Dehydrated beef	18.1	6		262	149	1.76	1.67–1.80

In experiment 1-a, no difference was found in the efficiency of utilization by rats of the proteins of raw cured pork and canned cured pork at either the 20 or 29% level of protein. At the 10% level of protein, a slightly higher efficiency of utilization was observed for the raw cured pork proteins, although inspection of the respective ranges of individual values indicates the difference may not be a real one.

In experiment 1-b, a cereal mixture fed with raw cured pork also showed a slightly higher efficiency of protein utilization than the same cereal mixture with canned cured pork. Although the range of individual values indicates less variation within these groups than at the 10% level above, the difference is very small if real.

In both experiments 1-a and 1-b, the values obtained for protein intake are apparent rather than absolute since they were calculated from the measured food intake with no correction for evaporation losses. The diets were of comparable moisture content and the protein efficiency values obtained should, therefore, be comparable, although they may run somewhat lower than values which would have been obtained if the absolute protein intake had been determined.

In experiment 2 the paired feeding technique was employed to eliminate the variations in protein intake observed in the previous ad libitum experiments. The data in tables 1 and 2 show no difference in the efficiency of protein utilization at the higher protein level. A statistical analysis (see Snedecor, '40) indicates that the difference between the protein efficiencies of raw cured and canned cured pork at the suboptimal level is slightly significant (probability of one in twenty that it could be due to chance). It is, therefore, probable that the canning process lowers the nutritive quality of the proteins to a small extent.

The uneaten food was assayed for moisture content in this experiment to permit measurement of the absolute food intake from which the absolute protein intake was then calculated.

In experiment 3, a comparison of the efficiencies of utilization of dehydrated beef and pork proteins showed a slightly

higher value for dehydrated beef at the suboptimal level. It is difficult to determine whether or not this may be a real difference, but it is definitely not a great one. There is no difference at the higher level of protein intake.

The diets were air-dry and the values obtained are, therefore, absolute. The values are quite similar to those obtained by Hoagland and Snider ('26) for ox and hog muscle using a similar method, i. e., growth of male rats for a 60-day period at 10 and 15% protein levels. Their values for ox muscle

TABLE 2

Paired feeding data for rats fed raw cured and canned cured pork shoulder—Experiment 2.

GROWTH PERIOD	PORK PROTEIN LEVEL	PAIR NO.	DIET	FINAL WEIGHT	INITIAL WEIGHT	WEIGHT GAIN	PORK PROTEIN INTAKE	GRAMS OF GAIN PER GRAM OF PROTEIN
Weeks	%			*(gms.)*	*(gms.)*	*(gms.)*	*(gms.)*	
10	9.7	1	Raw	191	62	129	62.4	2.07
			Canned	186	63	123	61.6	2.00
		2	Raw	200	69	131	67.1	1.95
			Canned	181	75	106	66.5	1.59
		3	Raw	216	59	157	64.9	2.42
			Canned	200	58	142	64.9	2.19
		4	Raw	178	56	122	54.6	2.23
			Canned	178	56	122	55.0	2.22
		5	Raw	211	57	154	70.3	2.19
			Canned	201	55	146	68.1	2.14
		6	Raw	165	48	117	52.5	2.23
			Canned	154	49	105	54.7	1.92
8	18.8	1	Raw	216	61	155	78.1	1.98
			Canned	205	62	143	83.2	1.72
		2	Raw	250	68	182	100.5	1.81
			Canned	275	70	205	100.8	2.03
		3	Raw	246	61	185	99.2	1.86
			Canned	224	62	162	94.6	1.71
		4	Raw	262	57	205	109.8	1.87
			Canned	306	59	247	108.0	2.29
		5	Raw	263	49	214	105.9	2.02
			Canned	245	51	194	101.8	1.91
		6	Raw	276	72	204	107.4	1.90
			Canned	277	65	212	108.3	1.96

at 10 and 15% protein levels were 2.55 and 1.7 respectively; for hog muscle the corresponding values were 2.46 and 1.87 respectively. Since their samples were air-dried below 60°C. for about 24 hours, probably a less severe process as regards proteins than dehydration, this comparison with their values may afford presumptive evidence that the dehydration process does not damage the proteins of pork and beef to any great extent.

The results of a comparison of the protein quality of canned cured pork shoulder, roast fresh pork shoulder, fried fresh pork shoulder, dehydrated pork and dehydrated beef in experiment 4 placed dehydrated pork first, fried fresh pork shoulder second, and the remaining three of equal quality when compared at a suboptimal level of intake. The differences at this level were not very large. At a higher and more practical level of protein intake no differences in protein quality were observed between the five meats.

DISCUSSION

Since the evidence obtained shows some probability of a slight damage to the proteins of cured pork shoulder by the commercial canning procedure described, this may be an indication that a more severe processing schedule might damage the pork proteins to a greater extent than this processing schedule.

These investigations have not determined whether the schedules used in canning other meats, these schedules often being more severe than for cured pork, are damaging to the respective proteins, and also whether the nutritional quality of the muscle proteins of other species is changed by heat treatment.

CONCLUSIONS

It is indicated by the efficiency of protein utilization for rat growth that: (1) The nutritive quality of the proteins of cured pork shoulder may be slightly lowered by a commercial canning process. (2) The proteins of dehydrated pork muscle and fried fresh pork shoulder are indicated to be slightly

superior in nutritive quality to those of canned cured pork shoulder, roast fresh pork shoulder, and dehydrated beef muscle.

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THE AVAILABILITY OF THE CALCIUM AND PHOSPHORUS OF DEFLUORINATED ROCK PHOSPHATE FOR THE RAT

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TWO FIGURES

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The critical shortage of bone meal and calcium phosphate has stimulated a search for other mineral supplements which would be a satisfactory source of phosphorus for animal feeding. Raw rock phosphates and superphosphates can not safely be used in animal feeds because of their high fluorine content. However, methods have been developed by which rock phosphates and superphosphates can be defluorinated. The fluorine content can be reduced to less than 0.10%, which is below the toxic level for a mineral supplement in the feeding of any farm animal according to Mitchell ('43). Because relatively little is known about the availability for bone growth of the calcium and phosphorus of defluorinated rock phosphates this problem has been studied.

Three defluorinated rock phosphates have been considered. The calcium, phosphorus and fluorine content of these products and of bone meal and secondary calcium phosphate are shown in table 1.

Products nos. 1 and 2 were defluorinated by a superphosphate procedure, that is, the rock phosphate was treated with sulfuric acid to form superphosphate and then the excess acid and the fluorine were driven off by heat treatment. Product no. 3 was defluorinated by a fusion procedure. In this process

silica is added to the rock phosphate and the mixture is fused to expel the fluorine.

Cannon ('38) concluded that the calcium of product no. 2 was well utilized by growing chicks. Fraser et al. ('43) in experiments with a fused rock phosphate such as product no. 3 found the phosphorus to be well utilized by rats.

TABLE 1

Analysis of the products used in the experiments.

MINERAL SUPPLEMENT	CALCIUM	PHOSPHORUS	FLUORINE
	%	%	%
Product no. 1	25.71	13.72	0.04
Product no. 2	28.22	12.49	0.05
Product no. 3	30.80	12.67	0.04
Steamed bone meal	33.58	15.00	0.04
Calcium phosphate	29.45	22.79	0.00

EXPERIMENTAL

In a preliminary experiment product no. 1 was compared with bone meal at a level which furnished 0.26% of phosphorus in the diet. The basal diet was essentially the same as that used by Schneider and Steenbock ('39), and contained 0.046% phosphorus and 0.016% calcium. Eight pairs of female littermate rats weighing approximately 36 gm. at 24 days of age were selected and pair-fed. After 35 days the rats were sacrificed. Both femurs were removed and as rapidly as possible freed from the adhering flesh. They were first weighed in water and then weighed suspended in air. Specific gravity determinations were made according to the following formula:

$$\text{Specific gravity} = \frac{\text{Weight of bones in air}}{\text{Weight of bones in air} - \text{weight of bones in water}}$$

The excised femurs were then allowed to dry for 3 or 4 days at room temperature and x-ray pictures were made. They were then dried at 100°C., extracted with anhydrous ethyl ether and ashed at 600°C. This procedure of analyzing the femurs was used in subsequent trials. The average results of these determinations as well as the growth data clearly

showed that bone meal was superior to product no. 1 for bone formation in the rat. The growth of all the rats was rather poor, however. This was thought to be due to inadequate protein in the basal ration.

The experiment was repeated using a basal ration modified to include some blood fibrin. The basal diet (table 2) contained 0.060% of phosphorus and 0.027% of calcium. In this experiment bone meal was compared with two levels of product no. 1 and one level of product no. 3. The mineral supplements were added to the basal feed to give diets containing 0.26% of

TABLE 2

Composition of basal diet.

	%
Sucrose	51.68
Egg white (cooked)	13.00
Fibrin (alcohol extracted)	5.00
Starch (cooked)	20.00
Vitab[1]	4.00
Wesson salt[2]	1.32
Wesson oil	5.00
Vitamins A and D concentrate[3]	....

[1] A rice bran concentrate from National Oil Products Company.

[2] Ca and P-free modification of Wesson's ('32) salt mixture.

[3] Four drops per rat per week, supplying 680 I.U. of D, and 5,000 I.U. of A. Product of the White Laboratories, Inc., Newark, N. J.

phosphorus and 0.46% of calcium. A higher level of product no. 1 was also added to give a diet containing 0.42% of phosphorus and 0.75% of calcium.

Ten trios of female litter-mate rats were used to test product no. 1 and seven pairs to study product no. 3. The growth data for the 35-day test and the average results of the analyses of the femurs are shown in table 3.

From these data it is clear that product no. 1 was inferior to bone meal. The rats fed bone meal made more growth and developed larger and more dense bones than those fed product no. 1. Even when product no. 1 was fed at an appreciably higher level it did not produce as good results as did bone meal. In sharp contrast product no. 3 permitted equal growth

and fully as large and dense femurs as when bone meal was fed.

In a third experiment it was decided to compare three defluorinated rock phosphates at four levels of intake. Secondary calcium phosphate was used as a standard. An experiment of factorial design was selected because it establishes a curve of response for each supplement and permits the simultaneous comparison of the effects of any level of one product with any level of another. The basal diet was the same as that used in the second experiment (table 2).

TABLE 3

A comparison of mineral products no. 1 and no. 3 with bone meal

SUPPLEMENT	NUMBER OF RATS	FOOD EATEN	GAIN IN WEIGHT	FEMUR ANALYSES			
				Specific gravity	Weight of femur ash	Ash content	Ash per cubic millimeter
		gm.	*gm.*		*mg.*	%	*mg.*
Product no. 1	10	262	60.8	1.26	106.5	55.0	0.34
Bone meal	10	262	73.0	1.44	200.4	64.1	0.55
Product no. 1 [1]	10	262	57.8	1.32	128.4	58.1	0.40
Product no. 3	7	277	75.4	1.44	191.1	63.2	0.55
Bone meal	7	277	77.6	1.47	202.8	64.0	0.57

[1] This diet contained 0.42% of phosphorus and 0.75% of calcium.

The phosphorus supplements to be tested were added in varying amounts to the basal mixture to give diets which contained 0.15, 0.25, 0.50 and 1.00% of phosphorus. The calcium content of the diets was adjusted with calcium carbonate so that all diets contained a Ca : P ratio of about 2 : 1.

One female and one male rat at 24 days of age were allotted at random to each of the diets. The average initial weight of the females was 34.3 ± 2.6 gm. and for the males 33.4 ± 2.1 gm. All animals were fed equal caloric amounts of the diets, adjustments being made if the rats began to leave food.

During the 35-day trial the rats on the lowest level of mineral supplement ate 227 gm. of their diet, while those on the higher level of mineral supplement received slightly more. The rats on the highest level of product no. 1 ate about 90%

of their food. The general appearance of all the rats was normal.

The average weight gains of the rats on the different supplements at the various levels of calcium and phosphorus intake are shown in figure 1. Although the gains were somewhat irregular, due to animal variation, it can be noted that the weight increase of the rats on calcium phosphate product no. 2, and product no. 3 were essentially the same. The curve of gain in weight for the rats on product no. 1 shows a downward trend, however, as the intake of this mineral was increased. This indicates that some factor was progressively

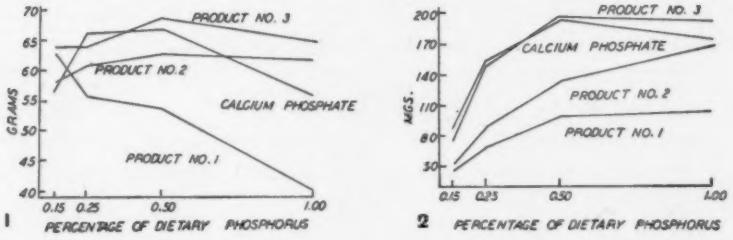

Fig. 1 The gain in weight of rats fed the mineral supplements at increasing levels.

Fig. 2 The total femur ash of rats fed the mineral supplements at increasing levels.

inhibiting growth as the level was raised. The depression of the growth of the rats due to product 1 was statistically significant.

The average total ash of the femurs for the male and females on the various diets at each level is presented graphically in figure 2. This criterion shows the greatest differences of all the measures used in this study. One observes that the femur ash for the rats receiving product no. 3 and calcium phosphate are almost identical, indicating the same utilization of these products at the various levels. It is observed that 0.25% phosphorus from product no. 2 gave about the same femur ash as 0.15% from calcium phosphate and that 0.50% of

phosphorus equaled 0.25% from calcium phosphate. Thus it appears that product no. 2 is only about one-half as available for bone formation as calcium phosphate. The femur ash of the rats receiving product no. 1 was definitely below all the others. At 0.50% of phosphorus product no. 1 approximately equaled 0.15% from calcium phosphate, thus indicating that product no. 1 was only about one-third as available for bone formation as the calcium phosphate.

The average per cent ash of the dry fat-free femurs is shown in table 4. Here one observes that product no. 3 supported about the same bone growth as calcium phosphate. The differences in the per cent ash between these products

TABLE 4

The percentage ash in the dry fat-free femurs.

SUPPLEMENT	LEVEL OF PHOSPHORUS				MEAN OF DIETS
	0.15	0.25	0.50	1.00	
	%	%	%	%	%
Calcium phosphate	53.55	61.84	65.25	64.42	61.27
Product no. 1	40.73	49.02	54.36	60.38	51.12
Product no. 2	44.28	51.39	60.86	63.74	55.07
Product no. 3	49.57	60.48	65.39	65.52	60.24
Mean of levels	47.03	55.69	61.46	63.52	56.92

are not significant. The femurs of the rats which received product no. 2 had a significantly lower per cent ash than did the femurs of the rats receiving the calcium phosphate. The rats receiving product no. 1 had the lowest ash percentage in their femurs.

Determinations of the specific gravity and the ash per unit of volume showed the same results as the other measures. The data are omitted in order to conserve space.

X-ray pictures were taken of the excised femurs in all of the experiments and in some cases full-body x-ray pictures were also made. This was done in order to obtain a further check upon the extent of bone calcification. The x-ray findings agreed with and supported the growth and bone data that are presented.

DISCUSSION

Both products no. 1 and no. 2 exhibited low availability for bone formation in the rat. The reason why these products were poorly utilized is not known. Both of these products were defluorinated superphosphates, and the presence of relatively large amounts of calcium metaphosphate may account for their low availability. MacIntire ('37) states that the phosphorus of raw rock phosphates exists as the ortho form. However, it is possible that calcium metaphosphates are produced during the superphosphate defluorination process.

Mitchell ('43) reports that the phosphorus of calcium metaphosphate prepared from rock phosphate is poorly utilized by the rat. Fraser et al. ('43) reports that the phosphorus of both calcium metaphosphate, c.p. and calcium metaphosphate produced from rock phosphate were poorly utilized by the rat. These workers did not state whether the calcium metaphosphate was amorphous or crystalline. MacIntire reports that in the production of calcium metaphosphate from rock phosphate one can get two types of metaphosphate depending upon the treatment. If the product is air-cooled a crystalline-like product of very low solubility results; whereas, if the hot product is quenched, a glassy-type, amorphous calcium metaphosphate which is much more soluble is obtained. It is possible that these two types of calcium metaphosphate differ in their availability to the animal organism, but the question needs to be studied.

It is suggested that the low availability of the defluorinated superphosphates, studied in these experiments, may be due to the presence of the lesser available calcium metaphosphate.

SUMMARY

A basal diet low in both calcium and phosphorus has been employed to study the availability of three defluorinated rock phosphates for bone formation in the rat. The rock phosphates have been studied at levels of from approximately 0.7 to 7.0% of the diet. These levels gave diets containing from 0.15%

phosphorus and 0.24% calcium to 1.00% phosphorus and about 2.0% calcium.

Product no. 1, a defluorinated superphosphate, was significantly less efficient for bone formation than calcium phosphate or bone meal. Product no. 1 inhibited the growth of rats, and this became more apparent as the level was raised.

Product no. 2, a defluorinated superphosphate, was less available for bone formation than calcium phosphate at low levels. However, when it was supplemented at approximately twice the level of calcium phosphate, equal bone formation resulted.

Product no. 3, a fused rock phosphate, was slightly less efficient than calcium phosphate at very low levels. When the diet was supplemented with this fused, defluorinated product to contain 0.50% phosphorus or more, however, product no. 3 was equally as satisfactory as calcium phosphate for bone formation.

It is suggested that the low availability of the defluorinated superphosphates, products 1 and 2, may be due to the presence of relatively large amounts of the poorly utilized calcium metaphosphate.

While these results may not apply to farm animals they suggest the importance of giving attention to procedures used in manufacturing defluorinated rock phosphates and of testing these products with farm animals.

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THE COPPER METABOLISM AND REQUIREMENT OF YOUNG WOMEN[1]

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ONE FIGURE

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The meagerness of the data available on the copper requirement of human beings is understandable when one remembers that only 15 years ago was copper added to the list of elements known to be essential to the animal organism.

One of the first suggestions as to the amount of copper needed for the maintenance of man was made by Tompsett ('34) as a result of metabolism studies conducted on some of his patients. One of these subjects on the very low copper intake of 0.21 mg. daily excreted 0.63 mg. per day. Since 0.63 mg. was needed to replace the amount lost, Tompsett suggested this amount as a minimum daily requirement. Chow and Adolph ('35) studied the copper metabolism of four normal adults for periods of from 1 to 3 days and found that equilibrium was reached on a daily intake of about 2.0 mg. of copper. Ohlson and Daum ('35) present figures for the copper intake and output of three young women for periods of 5 to 15 days in which the daily intakes ranged from 0.96 mg. to 1.15 mg. and in every case the excretion exceeded the intake by amounts from 0.08 to 0.40 mg. Orr ('35) made a dietary survey of the poorer classes in Aberdeen and used the presence or ab-

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sence of anemia as the criterion of adequacy of the copper content of the diet. In the non-anemic families the average daily intake of copper was 2.88 mg. and he suggested that the daily adult requirement was, therefore, no greater than 2.88 mg.

The data for the present report are from two sources: Ninety-five 1-week metabolism studies of sixty-five healthy young women who were living on their customary self-chosen diets, to be designated as series I; and a metabolism study of four healthy young women on an adequate constant diet for a continuous period of 75 to 140 days, to be designated as series II. The details of the set-up and procedure for the two studies respectively are reported by Leverton and Marsh ('42) and by Leverton and Roberts ('37). In both series composites were made of the food eaten by each subject and all excreta were collected for analysis.

Two colorimetric methods were used for the determination of copper, the one of Fischer and Leopoldi ('34) employing diphenylthiocarbazone and Coulson's ('37) modification of the Haddock-Evers method employing diethyldithiocarbamate. Comparison of results obtained by using both methods for the same materials showed excellent agreement. A photoelectric photometer was used for measuring the intensity or concentration of the color complex.

RESULTS

Because much of the value of the results lies in the number of subjects and, therefore, the cross section of metabolic activity that is represented for young women of this age, it is the plan to present average figures of all subjects and groups of subjects rather than figures of individual subjects. Throughout, the results of series I and series II will be presented together but will not be combined because series I includes many subjects for a short period whereas, series II includes a few subjects for a long period. The copper metabolism data for series I, sixty-five subjects on their customary self-chosen diet, and for series II, four subjects on an adequate constant diet, are given in table 1. The data have been sorted in two

TABLE 1

Copper metabolism data for all subjects and for different groups of subjects arranged according to the level of intake and according to the performance of the subjects.

DESCRIPTION	NO. STUDIES	NO. DIFFERENT SUBJECTS	AVERAGE DAILY				
			Intake	Coef. of variation	Excretion	Coef. of variation	Retention
			mg.	*%*	*mg.*	*%*	*mg.*
Series I							
Self-chosen diets	95	65	2.65±.0517	28.1	1.80±.0369	29.5	0.85±.0490
Séries II							
Constant diet	76	4	2.37±.0354	19.3	2.14±.0293	17.7	0.23±.0268
Data sorted according to size of intake							
Range of daily intake							
Series I							
1.00–1.99	16	15	1.60±.0328	19.6	1.57±.0653	24.7	0.03±.0560
2.00–2.99	51	38	2.48±.0272	11.6	1.71±.0455	28.3	0.77±.0388
3.00–3.99	23	20	3.42±.0377	7.9	2.10±.0778	26.4	1.32±.0739
4.00–4.99	5	5	4.21±.0428	33.6	2.11±.1655	26.0	2.10±.1369
Series II							
1.00–1.99	18	3	1.82±.0215	7.4	1.89±.0324	10.8	−.07±.0338
2.00–2.99	51	4	2.44±.0285	12.4	2.20±.0365	17.6	0.24±.0387
3.00–3.99	7	2	3.21±.0653	8.1	2.37±.0733	14.7	0.84±.1116
Data sorted according to performance of subjects							
Performance [1]							
Series I							
Negative	8	8	1.85±.1121	23.7	2.35±.1250	20.9	−.50±.0630
Equilibrium	9	9	2.22±.2167	40.9	2.19±.2155	41.1	0.03±.0149
Positive	78	56	2.78±.0517	24.2	1.70±.0311	23.8	1.08±.0410
Series II							
Negative	18	4	2.04±.0495	15.3	2.39±.0675	17.8	−.35±.0268
Equilibrium	10	4	2.03±.0575	13.3	2.01±.0535	12.5	0.02±.0095
Positive	48	4	2.56±.0131	16.6	2.08±.0332	16.4	0.48±.0314

[1] A storage or loss no greater than 5% of the intake was not considered significant and therefore was counted as equilibrium.

ways: first, according to the level of intake in order to compare the excretion and retention of copper at progressively greater intakes, and second, according to performance, that is whether the intake equaled, exceeded, or was exceeded by the excretion, thus indicating that the subject was in equilibrium, positive, or negative balance.

The average daily copper intake for all the subjects in series I was 2.65 mg., the excretion 1.80 mg. with the subsequent retention of 0.85 mg. In series II the intake was slightly less, 2.37 mg., the excretion slightly greater, 2.14 mg., and the retention, therefore, only 0.23 mg. The majority of the intakes in both series was between 2.00 mg. and 2.99 mg. of copper per person per day and on this intake some of the metal was retained. In series I, 82% of the studies showed the subjects to be storing copper and in series II, 63% of the studies showed copper storage. There were only eight cases in series I in which the amount of copper that was excreted in the urine and feces exceeded the intake, nine cases in which the excretion equalled the intake, and in the remaining seventy-eight cases the copper intake exceeded the excretion and there was an average daily retention of 1.08 mg. In series II there were forty-eight cases of copper retention, ten cases of equilibrium, and eighteen cases of negative copper balance of which fifteen were for the same subject; however, this subject was in copper equilibruim when the entire study was considered.

When the present data are sorted according to the size of the intake it may be noted that the excretion did not increase at the same rate as the intake and, therefore, there was an increased retention at each successively higher level of intake. The relation of the intake to the retention of copper is shown graphically in figure 1. For series I the $r = .6903 \pm .036$, and for series II $r = .5810 \pm .051$.

Of particular importance, if the results are to be used as a basis for determining requirement, is the relation between increases in intake and subsequent increases in retention. Expression of this relationship is somewhat involved, for it is not the percentage of the intake that is retained but the per-

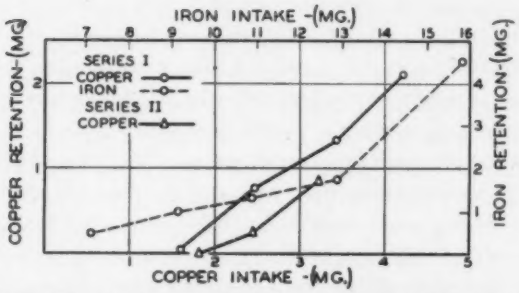

Fig. 1 The relationship between the level of intake and the retention of copper and iron for each series and of copper for series II.

centage of the increase in intake which is retained that may give the pertinent information. In table 2 are shown the increases in the average amounts of copper ingested and retained by groups of subjects at one level of intake over the average performance of the subjects in the group at the next lower level. The most significant column is the one which shows the percentage of each additional intake that was retained. It appears that a large proportion of each increase in copper intake was retained rather than unabsorbed or

TABLE 2

Relationship of increases in level of intake to increases in level of retention for copper and iron.

NUTRIENT	INCREASE IN AVERAGE DAILY INTAKE			INCREASE IN AVERAGE DAILY RETENTION			INCREASE RETAINED
	From	To	Actual	From	To	Actual	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*%*
Copper for series I	1.60	2.48	0.88	0.03	0.77	0.74	84.1
	2.48	3.42	0.94	0.77	1.32	0.55	58.5
	3.42	4.21	0.79	1.32	2.10	0.78	98.7
Copper for series II	1.82	2.44	0.62	−0.07	0.24	0.31	50.0
	2.44	3.21	0.77	0.24	0.84	0.60	77.9
Iron for series I	7.16	9.18	2.02	0.41	0.97	0.56	27.7
	9.18	10.87	1.69	0.97	1.34	0.37	21.9
	10.87	12.91	2.04	1.34	1.76	0.42	20.6
	12.91	15.85	2.94	1.76	4.55	2.79	94.9

excreted and that an increase in the intake to over 4.0 mg. still did not result in a diminished rate of absorption. The iron data for the subjects in series I which show the same high percentage absorption suggest the intestinal tract may treat the two metals in much the same manner.

The copper content of sixteen menses for the four subjects in series II was determined, and unlike the menstrual losses of iron by these same subjects (Leverton and Roberts, '36) varied greatly from one period to another. The average loss per period for each of the four subjects was 0.32 mg., 0.48 mg., 0.65 mg., and 0.74 mg. with an average of 0.55 mg. for all the subjects. The copper content of the menses determined by analysis was twelve times greater than when it was calculated from the volume of blood lost (calculated in turn from the iron content) and the average figure for copper content of blood. The subject with the largest copper losses in the menses had the lowest hemoglobin (12.3 gm. per 100 ml.) and the subject with the highest hemoglobin (13.5 gm. per 100 ml.) had the lowest copper losses. A third subject had an increase from 12.0 to 14.0 gm. hemoglobin over a 90-day period and during that time the copper content of the menses fell from 0.74 mg. in the first period to 0.49 mg. in the second, to 0.22 mg. in the third menstrual period. The fourth subject followed no discernible pattern.

No relationship could be found between the copper intake and retention and the hemoglobin values in the subjects of series I, and studies were not made during menstruation of the subjects.

The correlation between the intake of copper and iron, and between the excretion of copper and iron are as follows:

	SERIES I	SERIES II
Copper intake and iron intake	0.318 ± .0625	0.746 ± .0343
Copper excretion and iron excretion	0.380 ± .0595	0.517 ± .0567

There was no evidence that the level of copper intake influenced iron storage or vice versa. In series I the daily copper intake of the subjects who stored iron was 2.72 mg. as compared with 2.47 mg. by those who did not store iron, whereas

the daily iron intake of the subjects who retained copper was 10.40 mg., and 10.36 mg. by those who did not retain copper. It was not practicable to compare the intake of dietary essentials other than copper, for the group which was storing copper with the group which was not storing copper, because in the latter group there were too few subjects.

DISCUSSION

When results of a study of this kind are used in the determination of human requirement, major interest is focused upon the relation between the intake and the retention, whether positive or negative, of the nutrient under consideration. There are, however, limitations to the interpretation of even the most carefully conducted metabolism studies. Retention has to be calculated from the determination of the intake and of the excretion. Since the intake can be controlled the reliability of figures for retention is dependent upon how accurately the excretion reflects normal customary metabolic activity and how accurately the excretion is measured. There is considerable error inherent in the method of measuring fecal excretion, namely, that the carmine which is given to mark the stools is subject to the vagaries of intestinal action and motility, after which the analytical results depend upon the accurate separation of the marked from unmarked portion of the stools. For this reason small differences in results secured from studies of only a few days' duration must be viewed with extreme conservatism.

The figures for the retention of copper at different levels of intake and the upward trend of the curves shown in figure 1 suggest that the body's inability to excrete iron by way of the gut may also apply to copper. As the intake became progressively greater there was no flattening of the curves to denote a decrease in the proportion of the intake that was retained. Such a decrease could indicate that the current body needs had been met, that the rate of storage had approached its maximum and, consequently, that the unaccommodated copper was being excreted. Instead, the curve continues upward and at the

highest level of intake, much above the average for the majority, 50% of the intake was retained. There is, moreover, no hint that the curve will flatten at any level of copper intake attainable from ordinary foods.

Further evidence that the intestinal tract may handle copper and iron alike is suggested by the results which are plotted in figure 1 and illustrate the similarity between the intake and retention relationships for copper and iron for the same subjects. The actual amount of iron, as well as the percentage of the increase in the intake that was retained (table 2), is greater at each succeeding higher level of intake between 9.18 and 15.85 mg. of iron daily.

If the intestinal tract does not excrete copper, the occurrence of negative balances must have been apparent rather than real, and a result of such factors as: (1) too short a period of study, (2) unusual mixing of the carmine marker with the intestinal contents, (3) difficulty in separating the carmine-marked from the unmarked portions of the feces, (4) an atypical state of the subject, (5) an abnormal permeability of the individual's intestinal tract to the excretion of copper. Although reference to table 1 shows that the intakes of the subjects in negative balance in both series I and series II were smaller and the excretions larger than for the subjects in equilibrium or for those retaining copper, other information regarding them is also pertinent. The eighteen cases of negative balance in series II become insignificant when it is explained that fifteen of these occurred in the same subject and when the entire time of the study was considered she was in copper equilibrium and the other subjects were in positive balance. In series I there were eight cases of negative balance and these subjects had an average of 8.7 stools per subject per week, as compared with the 8.5 for those in equilibrium and the 8.0 for those who retained copper. The likelihood that negative copper balances are atypical permits discussion of some of the implications of the theory that copper is not normally excreted by the body.

If it is true that the intestinal tract permits only one-way passage of copper, and that in the direction of absorption, the

customary metabolism study technique for determining the requirement of a nutrient is inadequate when applied to copper. After this was found to be true of iron it was helpful to study how the body managed on very small amounts of iron (Leverton, '41), but this has yet to be done with copper.

With no indication of excretion of copper through the intestine the loss of copper from the body may be limited to urinary excretion and hemorrhage. This would suggest that the metal is used over and over again as is the case with iron, and, consequently, the requirement would be exceedingly small. The daily retention of 0.04 mg. would replace the urinary loss, which approximates 0.02 mg., and leave 0.02 mg. toward replacement of copper lost in the menses. The level of intake that would insure this theoretical amount of retention can be approximated from table 1. On self-chosen diets, many of which were found by Leverton and Marsh ('42) to be inadequate in respect to calcium, phosphorus, and nitrogen, the average daily intake of copper by all subjects was 2.65 mg. and this amount permitted an average daily copper retention of 0.85 mg.

In the sixteen cases with the lowest copper intake, 1.60 mg., there was a retention of 0.03 mg. and in fifty-one cases with copper intakes between 2.0 and 3.0 mg. (average 2.48 mg.) the daily retention was 0.77 mg.

Also reference to table 2 shows that 84% of the increased intake between the intakes of 1.60 mg. and 2.48 was retained. Considering these lines of evidence 2 mg. of copper would probably be an ample daily allowance for women of this age and certainly an allowance of 2.5 mg. of copper would be above question.

The chances are very good that self-chosen diets of even mediocre quality will contain 2.0–2.5 mg. copper because in the ninety-five studies of these subjects on self-chosen diets there were:

44 cases in which the daily calcium intake was less than 0.8 gm.
87 cases in which the daily phosphorus intake was less than 1.35 gm.
46 cases in which the daily iron intake was less than 10 mg.
78 cases in which the daily protein intake was less than 67 gm.

SUMMARY AND CONCLUSIONS

Data on the copper metabolism of young women are reported from two sources: Ninety-five 1-week studies of sixty-five young women on self-chosen diets, and a long-time study of four young women on an adequate constant diet.

The average daily intake by the subjects on the self-chosen diets was 2.65 mg. of copper and their average daily retention was 0.85 mg. For the subjects on the constant diet the average daily intake and retention of copper were 2.14 mg. and 0.23 mg. respectively.

As the copper intakes increased a large proportion of each increase was retained.

The copper content of sixteen menses for four subjects varied greatly from one period to another.

Considerable evidence is advanced in support of the theory that the body does not excrete copper and that the intestinal tract may handle copper as it does iron.

A daily allowance of 2.0–2.5 mg. of copper is suggested for young women together with evidence that this amount can be obtained from diets of otherwise mediocre nutritive value.

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FOLIC ACID, BIOTIN AND PANTOTHENIC ACID DEFICIENCY AND THE LIVER STORAGE OF VARIOUS VITAMINS IN RATS FED SUCCINYLSULFATHIAZOLE IN HIGHLY PURIFIED RATIONS

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The concentration in the tissues of various vitamins of the B complex has been employed by many investigators as an index of the adequacy of an animal's supply of these dietary essentials. Of the various tissues, changes in the vitamin content of the liver have, in general, shown the best correlation with variations in the vitamin intake and the nutritional status of the animal. In this communication data are presented on the storage of folic acid, biotin, and pantothenic acid in the liver of the rat, as influenced by the incorporation of succinylsulfathiazole in highly purified diets adequate in the well-recognized members of the vitamin B complex.

It has been shown (Welch, Mattis and Latven, '42; Welch and Wright, '43) that the inclusion of succinylsulfathiazole in amounts up to 10% of the ration has no demonstrable deleterious effect on rats when the remainder of the diet is composed of relatively crude ingredients (stock ration [1]). The inclusion of 1 or 2% of succinylsulfathiazole in rations composed of purified ingredients and containing thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, inositol, p-aminobenzoic acid, and choline in amounts believed to be

[1] The Purina brand was employed as a stock ration in these studies.

adequate, brings about a retardation in the rate of growth of rats. On such rations growth finally ceases and death occurs after a few weeks. Accompanying the effect on growth manifestations of dietary inadequacy such as alopecia, spectacled eyes, ophthalmitis, porphyrin-caked whiskers and achromotrichia may occur. A combination of crystalline biotin and folic acid concentrate [2] will effectively prevent or cure the condition. Reasons have been presented elsewhere (Black, Overman, Elvehjem and Link, '42; Martin, '42; Nielsen and Elvehjem, '42; Welch and Wright, '43; Wright and Welch, '43a) for relating the effect of succinylsulfathiazole to its effect on the synthetic activity of intestinal bacteria.

PROCEDURE

Young male black or piebald rats of approximately 50 gm. weight were caged individually over wide mesh screening and were fed ad libitum on the various rations outlined in table 1. After the animals had subsisted on these rations for varying lengths of time, representative animals were killed by decapitation and their livers assayed for several members of the vitamin B complex by microbiological methods. With some of the animals liver autolysates prepared by the method described by Wright et al. ('41) were used. In the remainder of the series the livers were subjected to enzymatic digestion with takadiastase at their natural pH [3] (Cheldelin et al., '42). In our experience autolysis yields approximately 90% (range 89–93%) of the pantothenic acid present in normal liver, based on the amount liberated after enzyme digestion with takadiastase. However, autolysis yields only 41% (range

[2] The substance termed "folic acid" is probably not a single entity. The evidence for the probable existence of several substances which can be utilized for growth by L. casei e or by Streptococcus lactis R, but which appear to be of significantly different potency, has been discussed by Stokstad ('43).

[3] Two gm. of liver fragmented with a spatula, suspended in 20 ml. water, takadiastase (equivalent to 2% of the liver weight) added, and digestion carried out for 18–24 hrs. at 37°C. under benzene. This, of several procedures, has given the highest values for folic acid in liver without the use of xanthopterin (Wright and Welch, '43b, 43c).

38–43%) of the folic acid obtainable from normal liver by takadiastase digestion. The biotin data were obtained from assays performed on samples of liver which had been digested by autoclaving at 15 pounds pressure for 1 hour with 6*N* H_2SO_4 and then neutralized with NaOH.

TABLE 1

Composition of diets employed in feeding experiments.

	s-5	s-6	s-6A	s-7	s-8
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Casein (Labco)	18.0	18.0	18.0	18.0	18.0
Fat (Primex)	...	10.0	10.0	10.0	10.0
Corn oil	2.0	2.0	2.0	2.0	2.0
Sucrose	72.4	59.9	59.9	59.9	59.9
Salts[1]	4.0	4.0	4.0	4.0	4.0
Cellu flour	1.5	4.0	4.0	4.0	4.0
A, D, and E concentrate[2]	..[4]	..[4]	..[4]	0.08	0.08
Choline chloride	0.1	0.1	0.1	0.1	0.1
Succinylsulfathiazole[3]	2.0	2.0	2.0	2.0	2.0
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Thiamine hydrochloride	3.0	..[5]	..[6]	0.2	0.2
Riboflavin	3.0	..[5]	..[6]	0.4	0.4
Pyridoxine hydrochloride	3.0	..[5]	..[6]	0.2	0.2
Nicotinic acid	2.5	..[5]	..[6]	4.0	4.0
Calcium pantothenate	2.0	..[5]	..	4.4	...
p-Aminobenzoic acid	...	...	..[6]	4.0	4.0
Inositol	...	...	..[6]	8.0	8.0
2-methyl-1, 4-napthohydroquinone diacetate	...	...	...	1.0	1.0

[1] Osborne and Mendel ('13) or Hubbell, Mendel and Wakeman ('37).

[2] Compounded as follows: fish liver concentrate containing 450,000 U.S.P. units of vitamin A and 90,000 U.S.P. units of vitamin D per gram, 7 gm.; α-tocopherol, 2 gm.; corn oil, 41 gm.

[3] In rations containing no succinylsulfathiazole this compound was replaced by an equivalent amount of sucrose.

[4] Animals in these groups were given 2 drops per week of percomorph oil fortified with α-tocopherol (1 mg. per 25 mg. oil).

[5] Animals in this group were given a daily subcutaneous injection of 1 ml. of a solution containing (per ml.) thiamine hydrochloride, 10 μg.; pyridoxine hydrochloride, 10 μg.; riboflavin, 20 μg.; nicotinic acid, 200 μg.; calcium pantothenate, 220 μg.

[6] Animals in this group were given a daily subcutaneous injection of 1 ml. of a solution containing (per ml.) thiamine hydrochloride, 10 μg.; pyridoxine hydrochloride, 10 μg.; riboflavin, 20 μg.; nicotinic acid, 200 μg.; p-aminobenzoic acid, 200 μg.; inositol, 400 μg.

In general one microbiological assay for each factor was performed on all the tissues of a given experiment. While there may be slight differences between the results of consecutive assays, all values within a single experiment are strictly comparable.

Riboflavin was determined by the method of Snell and Strong ('39). The determinations of pantothenic acid were carried out by either the method of Pennington, Snell and Williams ('40) or that of Landy and Dicken ('42). The methods of the latter workers were employed in the determinations of folic acid, biotin, and nicotinic acid. Titration of the lactic acid produced after a growth period of approximately 72 hours was employed as a measure of the response of Lactobacillus casei to the factor in question. Samples of crystalline biotin from two sources served as reference standards for biotin determinations. A folic acid concentrate [4] served as the folic acid standard. This was assayed against a sample of liver extract (Wilson's fraction B) and the results have been calculated in terms of micrograms of "potency 40,000 units," as described by Cheldelin et al. ('42).

RESULTS

Wright et al. ('41) and Mitchell and Isbell ('42) have presented data on the B vitamin content of normal rat tissues. We have found that the inclusion of succinylsulfathiazole in highly purified rations does not cause a significant deviation from the normal values for the concentrations of either riboflavin or nicotinic acid in the liver. The level of riboflavin in the liv of several series of sulfonamide-fed rats averaged 25 μg. per gram (range 18–27 μg. per gram). Nicotinic acid levels in several groups of animals, likewise receiving succinylsulfathiazole in highly purified rations, averaged 155 μg. per gram (range 119–189 μg. per gram). Since these observations indicate that no gross change in the composition of the liver occurred during the period of sulfonamide administration, they support the hypothesis that the storage of specific

[4] Kindly furnished by Dr. E. L. R. Stokstad.

B-vitamins in the liver may be used as a criterion for the estimation of the status of an animal with reference to the nutritive essentials measured.

Liver from animals fed purified rations adequate for normal growth contained only a fraction of the folic acid found in the hepatic tissue of animals subsisting on the stock ration. The inclusion of succinylsulfathiazole in the same purified rations (which rendered them inadequate for normal growth) resulted in only a slight further diminution in the amount of folic acid found in the liver. Animals which were fed rations adequate except that pantothenic acid was omitted, had a slightly higher concentration of folic acid in the liver than did those which consumed entirely adequate synthetic rations (in which neither succinylsulfathiazole nor folic acid were present).

The biotin content of the liver from animals maintained on the stock ration was found to be about 1.5 μg. of biotin per gram of fresh tissue. When the animals were reared on synthetic rations without added biotin, but adequate in the other well-recognized constituents of the B complex, the biotin content of the liver was considerably depressed, although evidence of a biotin deficiency was either absent or appeared only in a mild form after the animals had subsisted on such rations for several months. The inclusion of 2% succinylsulfathiazole in the ration caused a further depression in the amount of biotin found in the liver and signs of biotin deficiency were frequently evident (Nielsen and Elvehjem, '41). Neumann, Krider and Day ('43) have also studied the biotin deficiency which develops in rats fed a purified diet containing succinylsulfathiazole and p-aminobenzoic acid. Our data suggest that for signs of biotin deficiency to make their appearance in the rat, the biotin content must be reduced to approximately 0.35 μg. per gram of fresh liver. Two micrograms of biotin per day, administered subcutaneously, were found sufficient to maintain a concentration of biotin, in the liver of rats fed 2% succinylsulfathiazole, equal to that of animals on the stock ration.

The level of pantothenic acid in the liver was uninfluenced by the addition of succinylsulfathiazole to the extent of 10% in the stock ration. However, when succinylsulfathiazole (2%) was added to highly purified rations containing hitherto adequate amounts of pantothenic acid, low concentrations of pantothenic acid in the liver were invariably found. In such cases the content of pantothenic acid in the liver was consistently found to be comparable to the low levels encountered in induced pantothenic acid deficiency, produced by a ration (S–8) containing neither pantothenic acid nor succinylsulfathiazole. Changes seen in pantothenic acid deficiency (Unna, '40; Unna and Richards, '42) such as marked achromotrichia and porphyrin-caked whiskers, were noted when rats were maintained on purified rations to which succinylsulfathiazole was added (2%). Similar observations with regard to achromotrichia have also been described by Martin ('42), who employed sulfaguanidine. Evidence for pantothenic acid deficiency in rats receiving purified rations, low in protein and containing sulfapyridine, has recently been presented by West, Jefferson and Rivera ('43). These workers found that the oral administration of 1 mg. of calcium pantothenate daily caused more or less complete abolition of the signs of pantothenic acid deficiency within a period of 2 to 4 weeks. In our experiments in which the B-vitamins were given subcutaneously (diets S-6, table 1), neither the subcutaneous administration of calcium pantothenate (0.22 mg. daily), nor a marked increase in the amount of the vitamin in the diet (11 mg. per 100 gm.) raised the depressed pantothenic acid content of the hepatic tissue of rats fed succinylsulfathiazole in purified diets (table 2).

Our analyses of the pantothenic acid content of the livers of rats maintained on rations adequate for normal growth, except with respect to pantothenic acid, indicate that evidence of pantothenic acid deficiency in the rat may be anticipated when the concentration in the liver falls below about 50 μg. per gram.

TABLE 2

Pantothenic acid, biotin and folic acid in the liver of rats on various diets.

TABLE 2

Pantothenic acid, biotin and folic acid in the liver of rats on various diets.

EXPERIMENT NO.	SUCCINYLSULFATHIAZOLE	TYPE OF RATION	DURATION OF EXPERIMENT	PANTOTHENIC ACID	BIOTIN	FOLIC ACID
	%		*weeks*	*μg./gm.*	*μg./gm.*	*μg. "potency 40,000"/gm.*
1	..	S-5 Purified ration	4	145(110–201) (4)[1]	2.0(1.5–2.5) (4)[1]	1.0(0.6–1.7) (4)[1]
1	2	S-5 Purified ration	4	58(46–66) (4)	1.3(1.2–1.5) (4)	0.84(0.56–0.96) (4)
2	2	S-6 Purified ration; B-vitamins subcutaneously	6	41(39–53) (5)	0.89(0.72–1.1) (5)	.....
2	2	S-6 + 2 μg. biotin per day subcutaneously	6	.....	1.2(1.2–1.3) (3)	.....
2	2	S-6 + 16 μg. biotin per day subcutaneously	6	.....	1.6(1.4–1.8) (3)	.....
2	2	S-6A + 220 μg. Ca. pantothenate per day subcutaneously	6	45(42–50) (3)	.....	.....
2	2	S-6A + 11 mg. % Ca. pantothenate in ration	6	46(44–48) (3)	.....	.....
3	..	S-7 Purified ration	6	81(61–120) (5)	0.81(0.70–1.1) (5)	1.1(0.76–1.6) (5)
3	2	S-7 Purified ration	6	39(32–48) (5)	0.57(0.41–0.73) (5)	0.48(0.17–0.56) (5)
3	..	Stock ration	..	78(62–104) (5)	1.5(1.3–1.7) (5)	13(10–16) (5)
4	..	Stock ration	6	73(67–79) (4)	1.4(1.2–1.7) (5)	.....
4	10	Stock ration	6	78(73–88) (4)	1.3(1.0–1.7) (5)	.....
5	..	Stock ration	..	89(88–91) (4)	.....	20(14–26) (8)
6	..	S-7 Purified ration	8	96(93–99) (2)	0.41(0.40–0.42) (2)	2.6(2.6–2.6) (2)
6	2	S-7 Purified ration	8	44(35–49) (3)	0.29(0.20–0.40) (3)	1.6(1.5–1.6) (3)
6	..	S-8 Purified ration, no pantothenic acid	8	38(33–47) (3)	0.62(0.58–0.65) (3)	3.6(3.5–3.7) (3)
7	..	S-7 Purified ration	12	83(82–85) (2)	0.41(0.38–0.44) (2)	2.5(2.2–2.7) (2)
7	2	S-7 Purified ration	12	40(33–46) (3)	0.25(0.18–0.32) (3)	1.6(1.0–1.9) (3)
7	..	S-8 Purified ration, no pantothenic acid	12	46(44–49) (3)	0.79(0.75–0.84) (3)	4.6(2.4–7.0) (3)

[1] Accompanying the average values presented are (1) the range in values obtained, and (2) the number of animals studied.

Biotin extraction was done by acid hydrolysis; pantothenic acid and folic acid by enzymatic digestion, except in experiments 1, 2 and 4 where autolysis was employed. All data are based on the weight of fresh liver employed.

TABLE 3

Liver analyses and changes in weight of rats[1] on a purified diet containing succinylsulfathiazole (2%) given various supplements by stomach tube.

DAILY SUPPLEMENT	NO. OF ANIMALS	DURATION OF EXPERIMENT	CHANGES IN WEIGHT	PANTOTHENIC ACID CONTENT OF LIVER	BIOTIN CONTENT OF LIVER	FOLIC ACID CONTENT OF LIVER
		weeks	*gm.*	*μg./gm.*	*μg./gm.*	*μg. "potency 40,000 units"/gm.*
None	3	0	0	40(33–46)	0.25(0.10–0.32)	1.5(1.0–1.9)
None	3	3	− 8	48(47–49)	0.26(0.19–0.31)	1.2(0.72–1.6)
Crystalline biotin, 5 μg.	3	3	+ 16	57(55–61)	0.76(0.49–0.97)	1.2(0.96–1.5)
Crystalline biotin, 5 μg. and folic acid concentrate,[2] 20 mg.	3	3	+ 46	81(80–82)	0.89(0.81–0.94)	4.2(2.6–6.1)

[1] These animals had received for a period of 12 weeks the same purified diet (S-7) containing succinylsulfathiazole (2%).

[2] The concentrate of grass employed contained approximately 800,000 Snell-Peterson units of folic acid per gram. Biotin extraction was done by acid hydrolysis; pantothenic acid and folic acid were determined after takadiastase digestion of tissue samples.

Curative experiments demonstrate that concentrates of folic acid, administered with crystalline biotin, are effective in reestablishing excellent growth in rats which had previously reached a plateau in their growth curve, following the ingestion for several weeks of purified rations containing succinylsulfathiazole. The results obtained from one such experiment are presented in table 3. The folic acid concentrate employed was prepared from a grass juice powder [5] by a procedure similar to that described by Hutchings, Bohonos and Peterson ('41). Accompanying the resumption of growth and marked improvement in the general condition of the animals, which followed daily oral administration for 3 weeks of a concentrate of folic acid and crystalline biotin, there was a marked deposition in the liver of pantothenic acid, as well as of folic acid and biotin. The amount of folic acid and biotin found in the liver was intermediate between those amounts encountered in rats on synthetic rations adequate for growth, and those found in rats on stock rations. In addition, the simultaneous oral administration of folic acid concentrates and crystalline biotin to the deficient rats, increased the pantothenic acid content of the liver to the same level as that found in rats on the stock ration.

DISCUSSION

Data obtained from the assay of the hepatic tissue of rats receiving synthetic rations containing succinylsulfathiazole parallel the results of growth experiments, and the appearance of signs of deficiency, in that they show such animals to be deficient in both folic acid and biotin. In addition, a failure in the utilization of pantothenic acid has been demonstrated. Supplements of folic acid and biotin permit rats receiving succinylsulfathiazole to grow essentially normally for a period of several weeks (Nielsen and Elvehjem, '42; Martin, '42; Welch and Wright, '43) and folic acid concentrates, in the presence of crystalline biotin, not only cure the achromotrichia encountered in rats receiving sulfanilylguanidine (Martin,

[5] Supplied through the kindness of Dr. W. R. Graham of the Cerophyl Laboratories.

'42), but also cause a restoration of the utilization of pantothenic acid.

An explanation of this effect of folic acid concentrates and biotin is not yet possible. It is conceivable, however, that folic acid and biotin are involved in enzyme systems to which pantothenic acid is related. Pilgrim, Axelrod and Elvehjem ('42) found that liver slices from rats deficient in either pantothenic acid or biotin showed a decreased rate of pyruvate oxidation. It was concluded that the two factors are associated in metabolism.

Data presented in table 2 indicate that no impairment of pantothenic acid metabolism occurs when high levels of succinylsulfathiazole are included in a ration composed of natural crude materials. When a synthetic diet is fed, which contains succinylsulfathiazole, and an amount of pantothenic acid similar to that found in the stock ration, adequate hepatic storage of pantothenic acid fails, unless folic acid and biotin are given.

The question may be asked whether a certain type of bacterial activity in the intestine is essential for the proper utilization of "uncombined" pantothenic acid. Much information is available to indicate that pantothenic acid, as it occurs in animal and plant tissue (Cheldelin et al. '42), blood (Wright, '42), and yeast cells (Teague and Williams, '42), is largely in a combined or complex state. It is conceivable that folic acid and biotin are required, when succinylsulfathiazole is included in a highly purified ration, to maintain an intestinal flora which in some manner conjugates free pantothenic acid to an active complex or in some other fashion facilitates the utilization of this factor by the rat. It might be pointed out that the inclusion of zinc chloride in synthetic rations has been reported (Gross, Harvalik and Runne, '41) to produce signs in experimental animals closely similar to those encountered in pantothenic acid deficiency. Although the mechanism by which the effect is produced has not been explained, it is possible that the action of zinc chloride on the animal results from an influence on the intestinal flora. Such an effect might

be either on pantothenic acid or on the production of folic acid or biotin.

Molitor ('42) states that in the acute stage of induced pantothenic acid deficiency in the dog, the intravenous injection of pantothenic acid results in prompt recovery from the previously critical condition. Accordingly, on the basis of the evidence available at present, it is quite possible that folic acid and biotin produce their effects on pantothenic acid and other systems within the tissues of the animal and not through reactions occurring within the intestinal tract. A comparison of the minimal effective doses administered orally with those given parenterally may yield information bearing on the sites of action of these compounds.

However the effects of these factors are produced, it is clear that when folic acid concentrates and biotin are not administered to rats fed succinylsulfathiazole in highly purified rations, there develops decisive evidence of a failure properly to use pantothenic acid. In addition to a growth defect, black rats turn gray and present other characteristics of pantothenic acid deficiency not primarily because folic acid is a chromotrichial factor per se but because pantothenic acid metabolism is impaired when inadequate amounts of folic acid and biotin are available (Wright and Welch, '43a).

SUMMARY

A study of the storage of riboflavin, pantothenic acid, nicotinic acid, folic acid, and biotin in the liver was made in rats receiving various types of rations. A highly purified diet, adequate in those members of the vitamin B complex required for the production of excellent growth in rats, caused a marked reduction in the hepatic stores of folic acid and biotin compared with the amounts of these factors found in the liver of animals maintained in stock rations. The hepatic storage of these factors was further reduced by the incorporation of succinylsulfathiazole in such synthetic rations. The storage of riboflavin and nicotinic acid was not demonstrably influenced, a finding which indicates that no gross change in the liver

occurred during sulfonamide administration. Despite the presence in the diet of a previously adequate amount of pantothenic acid, the inclusion of succinylsulfathiazole caused a reduction in the pantothenic acid content of the liver to a level as low as that produced by a diet devoid of pantothenic acid. Increasing the dietary intake of pantothenic acid or giving the vitamin parenterally did not cause a renewal of growth, failed to modify the signs of pantothenic acid deficiency, and left unaffected the severely reduced amount of pantothenic acid in the liver. Administration of crystalline biotin and a concentrate of folic acid caused a prompt restoration of growth, recovery from the signs of pantothenic acid deficiency, and a restoration of the pantothenic acid content of the liver to normal. It is suggested that folic acid (or a constitutent of the folic acid concentrate employed) and biotin are essential for the maintenance of growth and of the general health of rats given purified rations containing succinylsulfathiazole. At least a portion of the effect of these factors is attributed to their playing an essential role in the utilization of pantothenic acid.

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SECONDARY ANEMIA DUE TO PROLONGED AND EXCLUSIVE MILK FEEDING AMONG SHOSHONE INDIAN INFANTS

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ONE FIGURE

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It has been found in a previous study that certain dietary deficiencies exist among Indians (Pijoan, Elkin and Eslinger, '43). Since numerous vicious circles of disease occur in many Indian children, it is one of the purposes of this communication to present data dealing with the first deficiency which makes its general appearance in Shoshone Indian infants. This deficiency is dependent on a continued and exclusive milk intake of from 1 to 2 years duration which results in an inadequate iron assimilation for hemoglobin formation. The other purpose of this communication is to present a modification of Elvehjem's method (Farrar, '35) of assaying iron in milk.

Recent studies by MacKay ('41) and by Merritt and coworkers ('34) indicate that a considerable part of the iron which disappears from the circulation of an infant during the physiological decrease of hemoglobin is retained for a variable period of time in body stores, but that within a period of a few months a supply of iron is needed for hemoglobin formation. Milk, on the other hand, is significantly low in iron. However, most infants have a sufficient iron reserve for the first few months which compensates for inadequate iron intake. After this period of compensation has transpired, the

relatively long continued feeding of an exclusive milk diet will produce an anemia primarily due to deficiency of iron (Elvehjem and coworkers, '33; Josephs, '34).

The subjects of this study were a group of healthy Shoshone Indian mothers who were admitted to the Western Shoshone Hospital, Owyhee, Nevada, for parturition and who were kept in the hospital for a period of investigation. They were then allowed to return to their dwellings and the studies were continued in some cases in the field. It must be said that in general the feeding of infants on the part of the mothers is from the breast until such time as the child can partake of the regular diet consumed by adults. There appears to be little supplementary feeding, if any, of vegetable purees, eggs or meat.

METHODS

Hemoglobin determinations were carried out on oxalated venous blood by use of the Evelyn and Cipriani method ('37), where the hemoglobin content of 15.6 gm. was equivalent to an oxygen capacity of 20.9 volumes, and represented an empirical hemoglobin value of 100%. In order to rule out primary blood disease, stained blood smears from each subject were studied.

Breast milk was obtained by the use of a breast pump. The milk was dehydrated and preserved for future assays. Elvehjem's method (Farrar, '35) was modified as follows: (a) elimination of iso-amyl alcohol and of potassium permanganate and (b) a change in volume of the other reagents. The elimination of iso-amyl alcohol is a reasonable one in view of the fact that no concentration of chromogen is required when sensitive color measurement apparatus is used; there appears to be no need in such a system of an oxidizing agent such as potassium permanganate. The modification that we suggest is relatively simple.

The reagents should be as iron-free as possible. If traces appear, the blank correction obviates a source of error. The first step is to obtain a calibration constant in which standards containing known amounts of iron are compared to a blank

containing the reagents. The blank is prepared as follows. To a 50-ml. volumetric flask add the following reagents: 5.0 ml. of 20% HCl, 1 ml. of 20% potassium sulfocyanate, 3 drops of conc. HNO_3, and make up to 50 ml. with distilled water. Ten milliliters of this solution is placed in an Evelyn apparatus (Evelyn, '36) and the galvanometer string is set at 100 mm.

An initial stock solution containing a known amount of iron is prepared and subsequent determinations are made on samples from this solution. Five-tenths gram of pure iron wire is dissolved in 20% HCl (sufficient to effect solution) and 1 ml. conc. HNO_3. The solution is evaporated to dryness and the residue is dissolved in 20% HCl, then diluted with water to 1000 ml. Each milliliter then contains 0.05 mg. Fe. Sample portions are then diluted to contain in each milliliter 0.00175 mg., 0.0020 mg., and 0.0015 mg. Each of these subsequent standards (1 ml.) is transferred to 50 ml. volumetric flasks and treated by the reagents used in the blank with the exception that 4 ml. of 20% HCl are used instead of 5 ml. An orange red color develops immediately and is stable for at least 2 hours. This chromogen is due to the formation of a complex salt as follows:

$$\text{HCl (in excess)} + \text{Fe}^{--} \longrightarrow \text{FeCl}_2 + (\text{HCl})$$
$$\text{FeCl}_2 + \text{HNO}_3 + (\text{HCl}) \longrightarrow \text{FeCl}_3 + (\text{HNO}_3) - (\text{HCl})$$
$$2\,\text{FeCl}_3 + 6\,\text{KCNS} \longrightarrow \text{Fe(Fe(CNS)}_6) + 6\,\text{KCl}$$

There appears to be sufficient proof (Schlesinger and Van Valkenburg, '31) that the final salt is $Fe(Fe(CNS)_6)$ rather than $K_3Fe(CNS)_6$ or $KFe(CNS)_4$.

Since the final chromogen has maximum optical densities in the neighborhood of 429 mμ, a Corning glass filter with transmission limits of 380 mμ to 460 mμ is used. The transmittance of the standard sample solution and that of the blank becomes a ratio. Since the galvanometer is adjusted to 100 with the blank, and the galvanometer reading is proportional to the amount of light transmitted, the following relationship exists: $T = \frac{G}{100}$ where T is the transmittance and G the galvantometer reading. Applying the laws of Lambert and Beer and where C equals the concentration of chromogen (or mg. of substance

responsible for the formation of the chromogen) and K_1 a calibration constant whose value depends on the nature of the chromogen and the characteristics of the filter (Malloy and Evelyn, '37), the following equation is used:

$$C = \frac{1}{K} \times \log_{10} \frac{1}{T} = \frac{1}{K} \times \log \frac{100}{G} = \frac{2 - \log G}{K_1}$$

Thus by determining the deflection caused in the galvanometer by light transmitted through standard solutions, the value for K_1 can be found experimentally. In our studies K_1 had a value of 0.1565 where the Fe content of milk would be:

$$\text{mg. Fe/liter} = \frac{2 - \log G}{0.1565}$$

The procedure of assaying the iron content of milk is as follows: 100 ml. of milk is placed in a platinum crucible, evaporated and initially carbonized in an oven (open door) at 110°C. Carbonization is completed in a muffle furnace at dull red heat. The ash is taken up in 20% HCl, and filtered through Whatman's ashless paper no. 40. The residue is reignited in a muffle furnace, taken up in 20% HCl and filtered. The filtrates are combined, then boiled in a water bath for 20 minutes and the volume kept constant by addition of 20% HCl. After cooling, the filtrate is transferred to a 50-ml. volumetric flask and made up to volume 50 ml. with water. One milliliter of this is transferred to another 50-ml. volumetric flask and treated in the same manner as the standard solutions. As a test of the accuracy of the method, known amounts of iron were added to milk samples which had been analyzed and subsequent determinations made. The average iron recovery in a series of seven samples was 98% of the theoretical recovery.

This modification of Elvehjem's method appears satisfactory, but it is well to point out that the color produced with sulfocyanate varies depending on the dissociation of the complex iron salt (personal communication from Dr. C. A. Elvehjem). We concluded from other experiments that this difficulty is almost completely eliminated by the use of standard volumes.

MILK AND HEMOGLOBIN STUDIES

Eleven lactating Shoshone Indian women were picked at random and the assays of iron in milk were as follows: 1.72, 1.80, 1.76, 1.59, 1.84, 1.72, 1.70, 1.72, 1.79, 1.72, and 1.78 mg. Fe/liter. Three women were also studied following parturition and hemoglobin determinations carried out on their children. The iron content of milk of the three women under special study is presented in table 1.

TABLE 1

The iron content of breast milk.

SUBJECT I		SUBJECT II		SUBJECT III	
Date	Fe	Date	Fe	Date	Fe
1940	*mg./liter*	*1940*	*mg./liter*	*1940*	*mg./liter*
10/12	1.84	11/4	1.71	12/4	1.84
10/16	1.91	11/8	1.68	12/8	1.78
10/20	1.72	11/12	1.74	12/14	1.84
11/1	1.74	11/16	1.82	12/20	1.70
11/2	1.78	11/20	1.72	12/30	1.62
11/10	1.82	11/30	1.74	1/10/41	1.74
11/15	1.78	12/4	1.70		
		12/20	1.74		

The foregoing data indicate that the iron content of milk is a fairly constant one and is in the neighborhood of 1.72 mg./liter. In 1928 Peterson and Elvehjem ('31) determined the iron content of milk and found it to be 2.4 mg./liter. We determined the iron content of the milk of four normal, healthy white women and obtained the following values: 2.3, 2.4, 2.3, and 2.4 mg./liter. Thus it would appear that in normal, healthy women of our own culture the figure is somewhat higher than that for the Shoshone Indian, even though the milk from the former is far from sufficient in iron to prevent the so-called "milk anemia" from developing. On the other hand the dietary of the Shoshone woman, high in refined cereal carbohydrate and fat and low in meat and vegetable proteins and iron containing foods, might conceivably play a role in reducing the iron content of maternal milk. Furthermore, the method of assay used gave a figure for cow's milk (2.4 mg.

Fe/liter) similar to that reported by Peterson and Elvehjem. On cow's milk and special feeding formulae where the iron content of the milk was found to be on the average of 2.4 mg./liter, a series of five infants maintained an anemic state, whereas those which received a supplement of iron showed extraordinary increase in hemoglobin. Such findings in other people have been noted before but were repeated in this study of the Shoshone Indian in order to obviate any possibility of

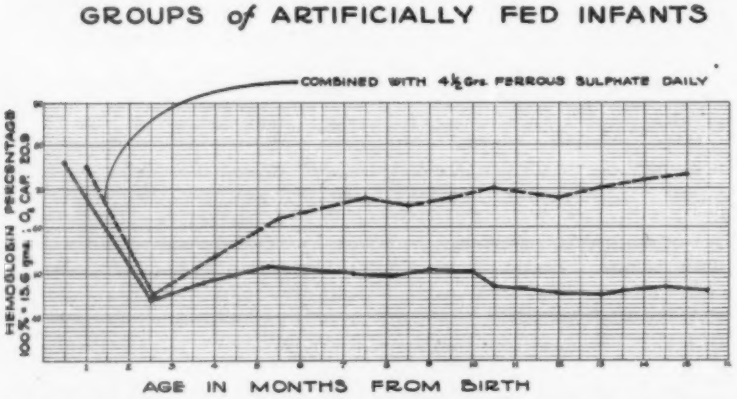
GROUPS of ARTIFICIALLY FED INFANTS
COMBINED WITH 4½ Grs. FERROUS SULPHATE DAILY
HEMOGLOBIN PERCENTAGES
100 % = 15.6 gms. ; O_2 CAP. 20.9
AGE IN MONTHS FROM BIRTH

Fig. 1 The figure represents two groups of infants, both having manifestations of anemia. One group was placed on a feeding formula which had an iron content of 2.4 mg./liter. The other group received 4½ gr. of ferrous sulphate daily. The first group continued to exhibit anemia, while the second group showed an appreciable increase in hemoglobin.

the anemia being due to other causes. Figure 1 represents two groups of five infants each where the limit of variation in each group in regard to hemoglobin content did not exceed 10%.

It would thus appear that iron is the limiting factor for hemoglobin formation in early infancy, all other things being equal. Hemoglobin determinations were also carried out on the children of the three mothers who were subjects of the milk iron study. The findings are reported in table 2.

From this table it would appear that the hemoglobin content at birth varies and the degree of red cell destruction and

anemia occurs at a variable period. No explanation for this is offered, especially in view of the fact that the iron content in the milk from the three mothers were within approximately the same range in content. Furthermore, the milk output in the three mothers did not differ appreciably (1440–1800 ml. daily).

Blood smears taken at weekly intervals from the children showed no unusual findings. Microcytes appeared during the fourth and fifth week, and from then on the blood picture was that of a secondary anemia.

TABLE 2

Hemoglobin content of blood in the three infants from the mothers studied.

INFANT FROM SUBJECT I		INFANT FROM SUBJECT II		INFANT FROM SUBJECT III	
Age	Hemoglobin	Age	Hemoglobin	Age	Hemoglobin
Days	*gm./100 ml.*	*Days*	*gm./100 ml.*	*Days*	*gm./100 ml.*
at birth	19.4	at birth	20.6	at birth	18.6
2	19.0	6	20.4	12	17.4
6	17.8	10	18.2	18	17.8
10	16.4	18	19.0	22	17.6
22	15.2	22	15.8	33	16.0
31	14.4	27	15.6	38	15.6
36	13.6	33	10.3	42	15.8
41	13.6	52	10.6	47	14.2
51	13.2	60	10.4	52	12.1
57	13.1	64	10.4	57	12.8
62	13.2	74	10.8	62	12.1
				67	11.4

DISCUSSION

The anemia resulting from an inadequate iron intake is significant in view of the fact that Rose and her associates ('30) found that an iron deficit occurred in a child who received 4.64 mg. a day. In such a case the iron output measured 5.74 mg., indicating a shortage in the food. Leichsenring and Flor ('32), Daniels and Wright ('34), Ascham ('35), and Porter ('41), in spite of some differences in their findings, advocate a liberal allowance in iron containing foods. On a milk regime the intake would be far from adequate (vide fig. 1) and inasmuch as the daily intake would never exceed 3 mg. from the total milk ingested, a low-iron anemia results.

Among Indians where many food habit deculturation processes have occurred, the tendency today is to breast feed the infant exclusively until such time as the child may eat of the food consumed by the rest of the family. Some Indians informed the investigators that in the past their parents used to chew food and then place this previously masticated food in the infant's mouth. Such a process was considered unhygienic by the invading culture and the practice was abandoned; it is, on the other hand, still continued by the Eskimo. One can assume that such a procedure might circumvent the effects of continued and exclusive milk feeding and thus obviate a low iron anemia.

The present problem is an interesting one and merits attention, for as the child continues in life (this is the subject of a separate communication) vicious circles are established wherein anemia, infection and certain avitaminoses mutually conspire against the body economy of these people. The problem is applicable to other Indian groups where continued breast feeding of an exclusive milk regime results in an anemia.

CONCLUSION

An anemia, secondary in character, is found among Shoshone Indian infants and is due to the continued and exclusive use of milk in their dietary. A modification of Elvehjem's method for determining the iron content of milk is introduced.

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BLOOD PLASMA ASCORBIC ACID VALUES RESULTING FROM NORMALLY ENCOUNTERED INTAKES OF THIS VITAMIN AND INDICATED HUMAN REQUIREMENTS [1]

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TWO FIGURES

(Received for publication July 30, 1943)

Ascorbic acid requirements for the human have been estimated over a wide age range by a variety of methods using differing criteria of adequacy and employing subjects with ascorbic acid deficiencies, subjects presenting other clinical problems, and subjects reported to be in a normal state of health. This latter group engaged in normal pursuits constitutes a sample of special value in establishing ascorbic acid requirements of individuals free from pathological symptoms.

The data which constitute present knowledge as to human requirements both in normal and pathological states have been obtained largely by urinary excretion studies, blood plasma analyses, and a combination of the two. Urinary excretion was early used as a means of evaluating human requirements. Refinement of techniques has made the measure of blood plasma ascorbic acid a rapid and valuable method for estimating ascorbic acid present for tissue use and adequacy of intake. Extensive use of this measure in population

[1] Part of the data in this paper was included in a thesis presented to the faculty of the Graduate School of the University of Tennessee in fulfillment of the requirement for the M.S. degree, June 1942 by Harvye Lewis.

surveys has tended to fix relationships between plasma levels and intake within rather narrow limits. Simultaneous studies of blood and urine have been made especially in studies of threshold and saturation levels.

Whatever the method used the primary objective has often been to determine the daily intake of ascorbic acid necessary to maintain saturation. Saturation itself has been variously defined but the underlying concept seems to have been that the least amount that is in excess is bound to be sufficient. In such studies, fixed intakes have been ingested over periods extending from 6 to 28 days; these periods have been preceded by intakes of 100–200 mg. to insure against tissue lack, terminated by test doses of 400–600 mg., and often succeeded immediately by another period of observation on measured intake. The test doses as well as the preliminary saturation intakes are greater than those of the average diet or those recommended. The assumption is that the body quickly readjusts to a state of equilibrium with the lower levels which are the object of study.

In this laboratory a survey study on 345 students has shown only partial correlation between plasma ascorbic acid value and the calculated amount of ascorbic acid in the previous day's dietary. Experiments conducted with eight subjects for a 2-week period on high intakes, 200–400 mg., failed to produce consistent or similar saturation levels in the plasma for the group or for individuals. An extended study on a single subject alternating high and low intakes of the vitamin failed to show a regular or immediate response. These findings throw doubt on fixed relationships between plasma levels and intakes.

The present study was undertaken for the purpose of observing blood plasma ascorbic acid values when ascorbic acid intakes were those ordinarily encountered. Intakes were maintained at each level for periods long enough to indicate adjustment. The change to suceeding levels was made without interruption by large test doses. Continuous observation of plasma ascorbic acid values on fixed levels should indicate

the tissue status with respect to ascorbic acid by a decrease, a gain or a leveling off of these values.

EXPERIMENTAL

Experimental data were collected from four subjects in 1942 and from eight in 1943 during January, February and March. The study was made at four levels of ascorbic acid intake. A synthetic vitamin preparation [2] was given daily in amounts of 25, 50, 75 and 100 mg. These amounts were augmented by the ascorbic acid of the basal diet which was essentially that of Belser, Hauck and Storvick ('39). A composite sample of foods which contributed ascorbic acid was analyzed daily. In 1942, the diet furnished 10.2 ± 0.47 mg. daily; in 1943, 6.9 ± 0.36 mg. daily. Intakes were, therefore, 32–35, 57–60, 82–85 and 107–110 mg. In 1942, the food was analyzed by the method of Woessner, Elvehjem and Schuette ('39), originally used for milk. In 1943, a modification of the Loeffler and Ponting ('42) method was used.

Foods containing ascorbic acid were eaten in the same amounts by all subjects. A daily dietary record was kept for each individual and checked with Recommended Dietary Allowances (National Research Council, '43). The subjects omitted rich sources of ascorbic acid from their diets for 2 weeks before the beginning of the experiment. They remained on each level of intake for 2 weeks; data on the 85- and 110-mg. levels secured for a third week in the first study gave no further significant information.

Fasting blood samples were taken each morning and analyzed immediately by the Mindlin and Butler micromethod ('38). During the last half of the second study 24-hour urine samples were collected daily from three subjects. These were analyzed by the method of Evelyn, Malloy and Rosen ('38) using Bessey's ('38) method of correcting for turbidity and color. At the close of the experiment all eight subjects were given a test dose of 400 mg. synthetic ascorbic acid and 24-

[2] "Cebione" was generously supplied by Merck and Company.

hour urine samples were analyzed for excretion of the vitamin to check the subjects by the criterion of saturation.

RESULTS OF PLASMA ASCORBIC ACID STUDIES

Plasma ascorbic acid values of all subjects showed daily fluctuations at every level of intake. Daily values are shown (fig. 1) for three subjects, 6, 7 and 11, who were representative

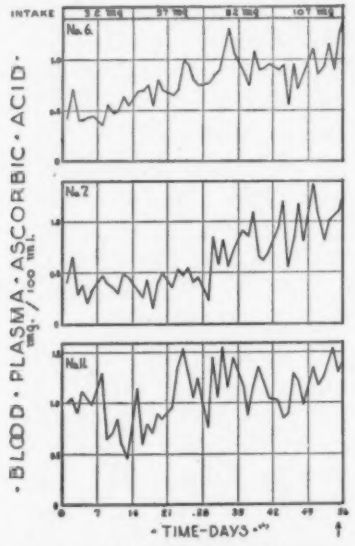

Fig. 1 Daily fluctuations of blood plasma ascorbic acid values for subjects 6, 7 and 11 at four successive levels of ascorbic acid intake. Arrow indicates intake of 400 mg. ascorbic acid.

of the group. The amplitude of the fluctuations and the time interval between successive high and low values tended to be quite regular and to a degree characteristic of the individual. On the 32- to 35-mg. intake level, successive upward swings gradually became less and downward swings dipped farther so that the general trend was a decrease in blood plasma values. As intake was increased this process was reversed. Two factors seemed reflected, rhythmic fluctuation and adjustment to different levels of intake.

An increase in intake did not immediately affect the fasting blood plasma ascorbic acid. Frequently the plasma value on the day following increased intake was decreased and was in general not typical of the intake but of the fluctuation. Values following the 400-mg. test dose at the close of the experimental period were within the range found on the 107- to 110-mg. intake. Adjustment to new levels came gradually. If weekly averages of daily plasma values are taken to indicate response to intake, definite trends can be observed (fig. 2). Holmes, Cullen and Nelson ('41) suggested that an average of a series of daily values gives a better estimate of the plasma ascorbic acid than single values taken at intervals.

The 32- to 35-mg. intake was characterized in all subjects by a decrease or bare maintenance of plasma levels. Individual differences did exist, however; extreme averages of 0.27 and 0.76 mg. % were found for the second week. This general decrease of plasma levels allowed subsequent changes with increased intake to take place uncomplicated by an accumulation of reserves.

The first week on 57–60 mg. generally resulted in plasma ascorbic acid values approaching those of the first week on the 32- to 35-mg. intake. By the second week all subjects showed increasing values with the exception of subject 5. Leaving subject 5 out of consideration, the extreme averages, 0.44 and 1.25 mg. %, would not normally have been expected on intakes identical for 4 weeks, yet the two represent the same dynamic picture, for this intake was satisfying normal body demands and furnishing a surplus which gradually appeared in the blood.

In eight of the twelve subjects the 82- to 85-mg. intake by the end of 2 weeks produced average plasma ascorbic acid values approximately equal to, or greater than those found later on the 107- to 110-mg. intake. For subject 5, this intake gave the first evidence of an amount great enough to supply body demands and to have an excess appear in the blood.

In the 1942 study no subject showed a rise in plasma ascorbic acid average due to the last 25-mg. increase in intake, while

in the 1943 study four of the eight subjects showed no rise. The other four subjects had not taxed the blood to the limit of its carrying capacity and at the end of 2 weeks at the 107– to 110-mg. level their plasma ascorbic acid averages were still mounting. After 8 weeks on identical ascorbic acid intake six of the eight subjects had blood plasma averages close to 1.0

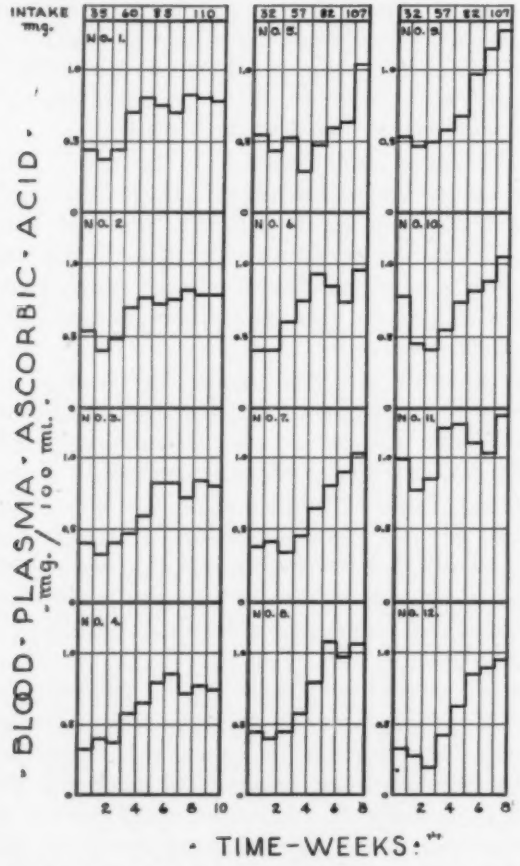

Fig. 2 Weekly averages of daily blood plasma values for each subject at four successive levels of ascorbic acid intake.

mg. %. In spite of this similarity of absolute values the behavior of individuals differed. One group was static and showed no tendency to alter plasma levels significantly. The other group was still building up the ascorbic acid content of the blood.

At the close of the experiment all subjects with the exception of 5, 9 and 10 were found to be saturated as judged by the urinary return of over one-half the test dose.

RESULTS OF URINARY EXCRETION STUDIES

Three subjects, 6, 7 and 11, were chosen for urinary excretion studies. During the first half of the experiment subject 11 had exhibited high plasma values, subject 7 low, while subject 6 gave values fairly typical of the group (fig. 1). Body weights of the three subjects differed. Subjects 7 and 11 were approximately the same age, while subject 6 was older.

Urine analyses were begun 2 days before the end of the 57-mg. intake and continued daily for the last 4 weeks of the experiment. Analyses were made on 24-hour samples collected after the first voiding in the morning. The results are shown in table 1. There was a daily fluctuation in excretion

TABLE 1

Average daily excretion of ascorbic acid for weekly periods at two levels of intake and retention related to body weight.

	PERIOD	AVERAGE INTAKE	AVERAGE EXCRETION, STANDARD ERROR	AVERAGE RETENTION	RETENTION PER UNIT BODY WEIGHT
	wk.	*mg./day*	*mg./day*	*mg./day*	*mg./kg.*
Subject 6	5th	82	22.8 ± 2.8	59.2	...
Wt.—61.8 kg.	6th	82	30.5 ± 4.8	51.5	0.83
Age—41 yr.	7th	107	38.0 ± 3.2	69.0	...
	8th	107	51.0 ± 2.0	56.0	0.91
Subject 7	5th	82	14.0 ± 3.1	68.0	...
Wt.—66.4 kg.	6th	82	10.0 ± 0.7	72.0	1.08
Age—23 yr.	7th	107	24.7 ± 6.2	82.3	...
	8th	107	35.7 ± 3.6	71.3	1.07
Subject 11	5th	82	25.8 ± 3.4	56.2	...
Wt.—51.4 kg.	6th	82	25.9 ± 2.1	56.1	1.09
Age—22 yr.	7th	107	39.5 ± 6.6	67.5	...
	8th	107	49.9 ± 2.0	57.1	1.11

as shown by the standard error, but weekly averages exhibited definite trends at the two levels of intake. Retentions were calculated for the second week at each level to permit adjustment to the 25-mg. increase of intake which appeared to be complete within 4 to 5 days. The saturation test made at the end of the experiment showed all three subjects to be saturated. Average retentions of ascorbic acid during the sixth and eighth week of experiment were in good agreement indicating that for these three subjects an 82-mg. intake was sufficient to supply normal body needs and provide an excess. An additional increase of 25 mg. was returned in the urine.

Retentions for subjects 6 and 11 were the same, while those for 7 and 11 differed. When related to body weight retention values for subjects 7 and 11 became 1 mg. per kilogram. For subject 6 who was older they were slightly lower. This finding is in keeping with reported data indicating high requirements for preschool children (Everson and Daniels, '36) and showing decreased concentration of ascorbic acid in the tissues of the human with age (Yavorsky, Almaden and King, '34). This retention or "utilization" of approximately 1 mg. ascorbic acid per kilogram of body weight approaches the lower values reported by others (Todhunter and Robbins, '40; Bryan, Turner, Huenemann and Lotwin, '41; Fincke and Landquist, '42) as the intake required to maintain saturation and was adequate to effect saturation in these three subjects.

DISCUSSION OF RESULTS

The trend of the data obtained during the 1942 and 1943 experiments was similar. Daily fluctuations in ascorbic acid values were a reality. They have also been reported by Storvick and Hauck ('42) and Roberts and Roberts ('42). Duplication of results was good; subject 2 of 1942 was subject 6 in the 1943 study, subject 3 was subject 7, and they exhibited similar values especially on the lower intakes. On the 32- to 35-mg. intake all average plasma ascorbic acid values were tending to decrease or had become stabilized. When the intake was increased to 57–60 mg. all subjects but one showed a

small surplus in the plasma which persisted throughout this period. In general, plasma values increased and reached a maximum during the 82- to 85-mg. intake. That all subjects might have reached a maximum at this level had the period been extended is open to question, but in the light of the urinary excretion data it is a possibility.

Single plasma ascorbic acid values or those taken at extended intervals give little information concerning the intake either as to absolute quantity or adequacy. The same values might be found at any two consecutive levels of intake; the extremes at any one level were frequently greater than the differences of weekly averages throughout the total range. Even weekly averages cannot be considered a satisfactory measure of intake; for example, those for the first 3 weeks were indistinguishable.

With increasing intakes of 32 to 110 mg., uninterrupted by large test doses, blood plasma ascorbic acid determined daily and for a long enough time to establish trends promises to give the clearest reflection of the system's requirements and adjustment to intake. If successive plasma ascorbic acid values are decreasing a deficit is being created. If they are increasing the system is utilizing what it needs and permitting a surplus to appear in the blood. If the values are definitely shown to be stabilized in the range characteristic of maximum blood levels the intake exceeds the requirements but the amount in excess cannot be judged.

The saturation test at the close of the 1943 study showed five of the eight subjects to be saturated on the basis of the return of over half the test dose. For subjects 5, 9 and 10 who were not, the adequacy of the intake to satisfy tissue needs is evidenced by the appearance of steadily increasing amounts of ascorbic acid in the plasma.

The urinary findings indicating adequacy at an intake of 1 mg. of ascorbic acid per kilogram of body weight when extended to trends shown by plasma averages of all subjects strengthen the relationship of intake to body weight. Subject 5, whose plasma values did not increase until intake rose to

82 mg., weighed 84.1 kg. Subject 11, whose plasma values started to rise on the 57-mg. intake and reached maximum on it, weighed 51.4 kg. All other subjects of intermediate weights, 55–69 kg., began to show plasma average increases on the 57- to 60-mg. level.

SUMMARY

1. A study of daily ascorbic acid requirements of the normal human adult has been made on twelve subjects on controlled, normally encountered ascorbic acid intakes of 32 to 110 mg., gradually increased without interruption by large test doses, over periods of 8 and 10 weeks.

2. The subjects were brought into equilibrium or slight deficiency as shown by plasma ascorbic acid averages which were slowly decreasing or only maintaining themselves. This was accomplished by an initial period on a 32- to 35-mg. intake of ascorbic acid.

3. Weekly averages of daily plasma ascorbic acid values gave a better reflection of the vitamin status of the individual than single determinations. Trends thus established showed the dynamic status of the individual with respect to intake.

4. Urinary excretion studies were made on three subjects for a 30-day period. "Utilization" values were shown to be close to 1 mg. per kilogram body weight. An intake of 1 mg. per kilogram of body weight was shown to increase plasma ascorbic acid values for all subjects studied and three subjects were shown to reach saturation on such a retention.

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ACTIONS OF BENZEDRINE AND PROPADRINE IN THE CONTROL OF OBESITY[1]

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ONE FIGURE

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Benzedrine and propadrine have been described as drugs which are useful in controlling body weight by numerous observers (Ulrich, '37; Lesses and Myerson, '38; Rosenberg, '39; Donley, '37; Schube, McManamy, Trapp and Myerson, '37; Myerson, '39; Fulton and Humphrey, '39; Ersner, '40), although these results have been denied, or the interpretations qualified, by others (Baker, '38; Dub and Lurie, '39; Cutting, '43). The usual explanation for such an effect is that these drugs control appetite. Other possibilities are an increase in energy expenditure from the well known excitant effects of benzedrine, modifications in carbohydrate metabolism, or impaired utilization of food through modification of the activity of the gastrointestinal system (Ulrich, '37; Lesses and Myerson, '38; Rosenberg, '39; Nathanson, '37; Davidoff and Reifenstein '37; Beyer, '39; Ersner, '40). A systematic study of these possibilities under laboratory conditions does not seem to have been carried out. Hence, it was thought worthwhile to determine if facts could be established which would help to explain the clinical effects, or lack of them.

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EFFECTS ON WEIGHT

Before the mechanism of action of benzedrine and propadrine in controlling obesity could be studied in the laboratory, it was necessary to demonstrate whether or not the loss of body weight was caused in animals as in patients.

Twenty white rats were fed 0.2 mg. of benzedrine in each gram of food for 3 weeks while living in revolving cages (Slonaker, '39). The growth was stopped and a slight decrease in weight occurred during the first week of medication, followed by a slow resumption of weight gain as tolerance developed. When the drug was withdrawn, there was a sharp increase in weight sufficient to make up for the loss, and failure to gain, during the period of drug-intake. These rats ran less than normally while medicated, so that increased exercise was not responsible for the effects on body weight. In another series with 7-day experimental periods, wide ranges of dosages of both benzedrine and propadrine were fed. While there were some irregularities due to individual variations in food intake and hence in drug ingestion, yet both drugs caused a decrease in body weight of up to 15% in 7 days. As little as 0.1% benzedrine caused a 9.2% loss, and 0.5% propadrine a 6.6% loss, indicating a rather marked effect in a low dosage range.

In another group 5 mg. per kilogram of benzedrine was injected subcutaneously twice daily for 8 days in five adult rats. The average weight dropped 6.5%. In other experiments to be described below where effects on weight were observed incidentally to other observations, large losses were also produced. Hence, these observations demonstrated that benzedrine and propadrine diminished the weight of white rats, when given in adequate doses either orally or by injection.

INCREASED ACTIVITY

One possible explanation of the weight loss was that the drug led to an increased energy expenditure.

It has been recently reported from this department (Schulte et al., '41) that benzedrine markedly increased spontaneous

movements as recorded in "jiggle cages", in doses of 0.3 mg. or higher per kilogram subcutaneously, and that propadrine also caused a slight stimulation but only in much higher doses. However, in the experiments above, propadrine produced substantially the same loss of weight as benzedrine, which would be inconsistent with the markedly different potency of these two drugs as control stimulants.

When benzedrine was injected subcutaneously in doses of 0.3 mg. per kilogram or propadrine 20 mg. or more in rats in revolving cages, a marked increase in running occurred which sometimes lasted over 7 hours (Tainter, in press). Here also there was a large discrepancy between the effects of benzedrine and propadrine. The above experiments were short-term observations, and hence did not reflect the total 24-hour changes. Therefore, attention was directed to feeding experiments in which the drug was mixed in the diet, and activity throughout the entire 24 hours recorded.

In feeding experiments in revolving cages of 7 days duduration, with 7-day control periods before and after, benzedrine caused a moderate increase in the daily revolutions in concentrations between 0.05 and 0.25 mg. per gram of food, which gave total intakes of approximately 10 mg. per kilogram daily. Higher doses depressed the activity. Propadrine increased the activity only in the range of 1.0 to 1.5 mg. per gram of food; lower concentrations were ineffective and higher ones depressed it. In a similar experiment on twenty rats, where the medication period was 21 days with correspondingly longer control periods, 0.2 mg. of benzedrine per gram of food gave an average intake of 18.1 mg. per kilogram daily. The activity decreased from about 7500 revolutions daily to 4700 on the sixth day, and then steadily recovered to normal as the rats developed a tolerance to the drug. The coincidental effects on body weight were described above. Obviously, the decreased activity could not explain the weight loss, since inactivity would conserve energy rather than the reverse.

These results, therefore, failed to demonstrate an increase in motor activity which might explain the loss of weight. However, since the criteria used were muscular movements of the animal, rather than oxygen consumption, it was conceivable that there might be changes in total metabolism which would not be reflected in activity determinations, but which could be measured from oxygen use over adequate periods of time. Therefore, the effects of benzedrine and propadrine on the oxygen consumption were studied.

OXYGEN CONSUMPTION

The first observations were on the effects of subcutaneous injections on the oxygen-use recorded hourly for 7 hours in the apparatus described by Van Winkle ('42). Three rats were studied simultaneously each day. Nine rats were used for the entire study, determinations being made repeatedly on each at intervals of 3 days or more, so as to compare the effects of the drugs in the same animals. In two control series, (table 1) all the rats were injected with physiological salt solution to measure the normal oxygen use after handling and injection of the animals. On other days various doses of benzedrine or propadrine were injected. The oxygen used each hour was observed, corrected to standard pressure and temperature, and calculated as milliliters of oxygen used per 100 cm.^{2} of body surface. Since the peak effect was invariably during the second hour, the average values for that hour are given in table 1 together with the percentage changes. The total metabolism was also calculated for the 7-hour period, and the percentage changes from the drugs computed.

There were some irregularities in the averages from spontaneous variations in activity of the rats. However, the data demonstrated that benzedrine caused a marked increase in oxygen use. In fact, the total effects from the 8 and 10 mg. doses were not recorded because the metabolism was still elevated about 25% at the end of the 7-hour observation period. Propadrine did not change the oxygen use, the small de-

viations from the control values being well within the limits of variation. These results closely agree with the control values reported by Van Winkle, using the same apparatus, and also with his results with injection of benzedrine (Van Winkle, '42).

Benzedrine has been observed by others also to increase the oxygen use in acute experiments (Delaunois and Marri, '39; Kleitman, '39; Dill et al., '39), and there is no doubt that such must result from the marked muscular movements produced by adequate doses of the drug. However, conclu-

TABLE 1

Effect of subcutaneous injection of benzedrine and propadrine in white rats on the oxygen consumption in acute experiments.

DRUG	DOSE MG. PER K.	NO. OF EXPTS.	AVERAGE METABOLISM IN SECOND HOUR		AVERAGE TOTAL METABOLISM IN 7 HOURS	
			In ml. O_2 per 100 cm.^{2}	Increase in percent of control	In ml. O_2 per 100 cm.^{2}	Increase in percent of control
Control		18	84.5		572.3	
Benzedrine	1	9	101.4	20.0	609.0	6.4
sulfate	2	9	113.6	34.4	653.4	14.2
	4	12	138.0	63.3	759.7	32.7
	6	18	114.4	35.4	716.6	25.2
	8	9	129.7	53.5	811.3	41.8
	10	9	122.6	45.1	815.7	42.5
Propadrine	1	9	85.0	—5.3	618.9	8.1
Hydrochloride	5	9	84.7	0.2	555.7	—2.9

sions could not be drawn as to the mechanism of weight loss unless it could be shown that the total oxygen use was increased over a long period of time, rather than temporarily.

Accordingly, continuous observations for weeks were made on the total oxygen used by individual rats, when fed the normal diet as well as food containing various concentrations of benzedrine. The rats lived in the metabolism chambers continuously, and their total oxygen use was recorded. The chambers were opened briefly once a day to permit cleaning, weighing, and feeding, and the total daily oxygen was corrected for this short interval. Because room temperature fluctuations modify the oxygen use, the average tempera-

ture of the room was recorded, and periods of medication were alternated with periods of normal diet of several days each. The 24-hour oxygen volume was corrected to standard pressure and temperature and calculated as liters of oxygen in 24 hours per 100 cm.^{2} body surface. The control values of each rat were used to calculate its regression line of metabolism against room temperature by the method of least squares. Deviations from this normal curve under the influence of the drug were calculated as percentage changes from the normal for that temperature. This is substantially the technique used by Van Winkle in his acute experiments (Van Winkle, '42). Since injections of propadrine had failed to change the metabolic rate even temporarily, these observations were made with benzedrine only.

In an individual animal under control conditions, daily deviations from the calculated normal metabolism averaged approximately 5%. To obtain a more general expression of the relationship of room temperature to oxygen use, the values for 100 control days on the seven rats were pooled and the regression line calculated. The formula was found to be $y = 3.70 - 0.51x$, where y is the oxygen used and x is the avearge room temperature. This covered the observed temperature range of from 19.6°C. to 27.1°C. within which limits the regression line was substantially linear. Because the grouped data were obtained from a relatively small number of animals differing in their individual rates of metabolism, the deviations from the regression line averaged 7% or $\pm$ 0.18 liter out of an average metabolism of 2.57 liters of O_2 per 100 cm.^{2} in 24 hours. The standard error of the y value was $\pm$ 0.238 liter or 9.3%. Benzedrine was mixed in the food for a total of 90 days in concentrations between 0.01 mg. and 1.2 mg. per gram, giving intakes ranging between 0.63 and 99.6 per kilogram daily. The resulting metabolic rates are plotted in figure 1. There was no indication of an increased metabolism from benzedrine, except in rats ingesting the nearly fatal amounts of 50 mg. per kilogram per day or more.

This result is in marked contrast to the increase from even small single injections of benzedrine during the first hours after administration. If the lack of apparent effect were due to development of tolerance, there should have been the greatest metabolism from any given dose during the first day of administration with lesser effects as the tolerance was built up. However, regrouping the data according to the day of administration showed that the metabolic responses to a given concentration were as great, if not greater, on the second and third days as on the first day.

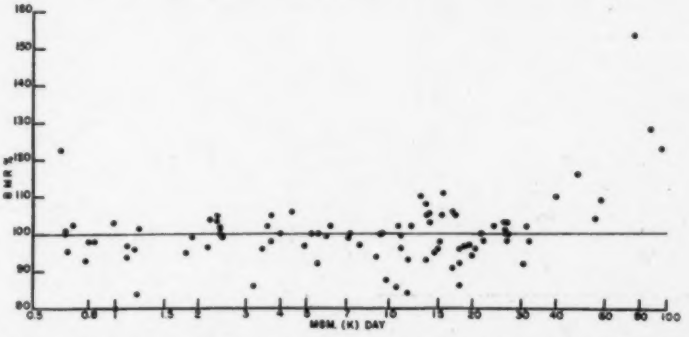

Fig. 1 Effects of various doses of benzedrine fed in the food on the oxygen use of white rats. Benzedrine doses are in milligrams per kilogram per day, and oxygen used in liters per 100 cm.^{2} body surface per day.

These data show that, while benzedrine may stimulate oxygen use for approximately 6 hours after administration, the increased metabolism is compensated for by subsequent decreased energy output, so that the total 24-hour metabolism is not increased, except by doses approaching the fatal. This is similar to the observations of Searle and Brown ('38), who found that benzedrine redistributed the activity output, but did not modify its average 24-hour level.

PAIRED FEEDING EXPERIMENTS

Paired feeding studies were next carried out with benzedrine and propadrine. For each, five litter mate pairs of rats

were used, housed in individual cages. Normal food was given freely for 13 days and 21 days, respectievly, before the drug was mixed in the diet, after which the food given daily to each control rat was equal to that eaten by the other member of the pair on the preceding day. Since the control rats did not always eat the food supplied them, they had a slightly smaller average food intake than those getting the drug.

TABLE 2

Results of adding benzedrine and propadrine to the diet. This represents two paired feeding series, in each of which five litter mate pairs were used. The figures in parentheses are the values for the control rats.

DAYS OF EXPT.	DRUG	CONCENTRATION OF DRUG IN FOOD	AVERAGE FOOD INTAKE	AVERAGE INITIAL WEIGHT	AVERAGE CHANGE IN WEIGHT
		mg./gm.	*gm./rat/day*	*gm.*	*gm.*
0–13	0	0	12.3 (12.7)	76 (76)	+ 37.8 (+ 33.4)
14–16	Benzedrine	0.6	9.2 (8.5)	113.8 (109.4)	— 6.8 (— 3.4)
17–48	sulfate	0.6	14.7 (12.5)	107.0 (106.0)	+ 39.6 (+ 56.2)
49–70		1.2	10.7 (10.2)	146.6 (162.2)	— 15.0 (+ 4.2)
71–92		2.4	9.1 (8.6)	131.6 (166.4)	— 11.6 (+ 15.1)
93–140		2.4	13.3 (11.5)	120.0 (181.5)	+ 21.0 (— 0.5)
0–11	0	0	8.2 (7.6)	38.0 (37.4)	+ 35.4 (+ 32.4)
12–21	0	0	10.0 (10.4)	73.4 (69.8)	+ 26.8 (+ 31.4)
22–30	Propadrine	2.4	9.2 (8.9)	100.2 (101.2)	+ 7.6 (+ 8.2)
31–40	hydrochlor-	2.4	10.7 (10.8)	107.8 (109.4)	+ 11.0 (+ 18.2)
41–59	ide	2.4	10.3 (10.3)	118.8 (127.6)	+ 16.8 (+ 30.6)
60–103	0	0	13.1 (12.5)	135.6 (158.2)	+ 57.2 (+ 51.0)

In the benzedrine series during the control days before medication, the gain was nearly equal on an almost equal food intake (table 2). For several days after 0.6 mg. benzedrine per gram was added to the food, the food consumption dropped sharply, and the medicated rats lost 6.8 gm. in weight. Their controls consumed even less food but lost only 3.4 gm. in weight. As the rats developed tolerance to the appetite effects of the drug, the food consumption increased, but the medicated rats gained less than did their unmedicated pairs, even though their net food intake was greater. This same sequence was reported each time the

benzedrine concentration in the food was increased. During the entire period, the medicated rats ate an average of 12.4 gm. as compared to the 11.0 gm. of their mates, but only gained an average of 27.2 gm. as compared to 71.6 gm. for the others. Obviously the medicated rats were much more affected than could be accounted for by simple diminution of food intake.

In the propadrine series there was a control period for 3 weeks before medication was started (table 2). There was a sharp decrease in the food intake to 6 gm. in the beginning of propadrine administration, but the appetite quickly returned, so that the average decrease in food intake during the first 8 days of medication was only to 9.2 gm. from the control of 10.0 gm. The differences in weight gain were not as marked with this dose of propadrine as with the benzedrine, but the same qualitative effects were present. Thus on an average food intake of 10.2 and 10.1 daily respectively, the medicated group gained an average of 25.4 gm. as compared to 57.0 gm. for the controls.

The mechanism by which this differential effect is brought about is uncertain. The most probable one is that the doses of benzedrine ingested were high enough to increase the metabolic level, since they were possibly in the stimulant range (figure 1). However, this thesis is difficult to sustain, since propadrine apparently does not increase metabolism nor activity in a manner similar to benzedrine. But, on the other hand, the changes from propadrine summarized in table 2 are so small as to be of uncertain probative value. If the energy metabolism of the medicated rats is not increased over the normal, the converse may occur, due to control rats having a decreased metabolism from the partial starvation. To decide between these hypotheses would require paired feeding experiments in metabolism chambers, which, unfortunately, could not be carried out at present. This phase of the study, therefore, must be deferred until some future time.

EFFECTS ON FOOD UTILIZATION

Because of the many reports that these drugs may relax the gastrointestinal tract and overcome spasm on the one hand, or cause stimulation on the other, the speed of passage of food through the gut was studied to determine if there were any changes which might impair the utilization or digestion of the food.

Twelve rats were selected for this purpose, each being used repeatedly at intervals of 4 to 7 days for a total of 180 tests, to compare the changes from the drug with numerous control observations. Food was removed from the cages the afternoon before the test. The next morning each animal was put into an individual cage in which there was powdered food containing 0.24% carmine. The animal was allowed to eat this food for 1 hour, during which time between 1 and 5 gm. were usually consumed, and then the colored food was replaced by normal diet. The drugs were mixed in the colored food, in concentrations from 0.3 to 2.4 mg. per gram, or were injected subcutaneously at the time the colored food was withdrawn from the cage. The stools of each rat were collected as passed in test-tubes of 1% ammonium hydroxide solution. The ammonia leached the red carmine color from the stools, without breaking them down or extracting the other pigments, and thus afforded an easy way of detecting the time of appearance of the carmine. Each rat was observed continuously until the stools became positive, and the time of passage of food mass thereby determined in hours from ingestion. Days of medication were alternated with control days to afford a reliable estimate of the normal times.

Inasmuch as the rats did not eat the same amount of food each day, it was necessary to establish whether the volume consumed would affect the passage time. Therefore, in control experiments, predetermined amounts of food were fed covering the range of amounts ingested during medication, and the normal times of passage averaged for different levels of food intake. At no level was there a significant deviation from the average of the entire series, nor was there any

tendency for a progressive change of speed with differences in amount of food ingested. Hence, the amount of food eaten did not affect the time required for it to pass through the gastrointestinal tract.

The average control passage time was 5.7 hours. When benzedrine was mixed in the diet, the animals receiving up to 5.0 mg. per kilogram, showed no change in the time. In the next group, where the average dose was 7.0 mg. per kilogram, there was a small but definite slowing to an average of 6.9 hours. This increased progressively with increase in dosage until at about 100 mg. per kilogram, there was apparently complete ileus for about 14 hours.

This slowing of peristalsis might be either a local effect of the drug directly on the wall of the gut, or a systemic action after absorption. Accordingy, in another series of rats, the benzedrine was injected subcutaneously instead of being fed. One-half mg. prolonged the time definitely, while 1.0 mg. and 2.0 mg. per kilogram increased it to about 11 hours. Similar observations with propadrine showed that it had at least as great an effect as the injected benzedrine, using doses of 0.5 and 1.0 mg. per kilogram subcutaneously. Obviously the slowing of peristalsis was much greater from injection of a given dose than from ingestion in food, which disposes of the possibility of there being a significant local effect on motility. Probably a part of the inefficiency of oral administration arises from delayed absorption as a result of the vasoconstrictor action of the drug on the intestinal mucosa.

The slow passage of food through the gut was undoubtedly accompanied by weak and inefficient peristalsis, and hence possible incomplete mixing with digestive enzymes and poor digestion. However, there was the opposite possibility that the prolonged time might have afforded a sufficiently longer interval for the completion of the chemical changes that there was no impairment of digestion and absorption. Evidence on this could be obtained by weighing the stools to see if there were a change in either direction which might throw light on these possibilities.

At this time, observations were being made of the effects of benzedrine on voluntary activity in rats, the results of which are being published separately (Tainter, in press). Since food intake, etc., were being measured, the stools of twenty rats were collected and weighed each day after drying at 110°C. If there were any undigested residue it would have increased the weight of the stools. During the 25 control days before medication, the dry stools weighed an average of 11.4% of the food intake. Benzedrine, 0.2 mg. per gram of food, was added to the diet giving an average dose of 18.1 mg. per kilogram daily, a dose definitely effective in changing intestinal motility. The dry weight of stools during the 21 days of this medication averaged 10.4% of the food intake. During the post medication (control) period of 22 days the weight of the dry feces was 10.3% of the food consumed. For the combined control periods, the average of 10.8% for the dry weight of the feces agreed closely with the 10.4% for the benzedrine-period. Hence, there was no evidence that the delayed passage of food through the gut was associated with changes in digestion or assimilation. Attention was, therefore, directed to the effects of benzedrine on food intake.

EFFECTS ON FOOD INTAKE

Data were available in many of the preceding series of experiments on the effects of benzedrine and propadrine on food intake or appetite. In a previous study (Tainter, in press) the average food eaten during 3 weeks of benzedrine and propadrine administration was compared with the corresponding non-medicated control periods. It was proved that any impairment of appetite was not due to the bitter taste of drugs in the food, since addition of other bitters did not decrease the appetite, but rather increased it, if anything. The average food intake for the twenty rats in the week preceding the drug was 93.8 gm. per kilogram daily. On an average daily dose of benzedrine of 15.4 mg. per kilogram during the first week, the food intake was decreased to an average of 77.3 gm. per kilogram daily. Thereafter a toler-

ance to the appetite effects of the drug developed so that, during the second week, the food intake had returned to within the normal range.

At first the average decrease in food eaten was 2.3 gm. per rat daily, which was accompanied by an average daily loss of body weight of 0.5 gm. The food had an energy value of 3.5 calories per gram, so the total deficit in caloric intake was 8.05 calories a day. Since animal fat has a caloric value 9.4 per gram, this decrease in caloric intake could have been made up by burning 0.85 gm. of the rat's fat, which, since fatty tisses are approximately 15% water, would have caused a loss of weight of 1.0 gm. a day. It is seen that the decrease in food intake was adequate to explain the observed loss of weight.

In another series, five rats were injected twice daily with 5 mg. of benzedrine per kilogram for 8 days, and lost an average of 15.6 gm. each. The average control food intake was 15.5 gm. a day, which the drug-injections decreased by 4.9 to 10.6 gm. a day. Such a decrease in energy intake could have been replaced by burning 17.1 gm. of fatty tissue. This checks well with the observed loss of weight of 15.5 gm. so that the diminished food intake would appear to be an adequate explanation of the loss of weight in this series also. It might be pointed out that, since the benzedrine was injected in these experiments, the effects on appetite were systemic rather than local on the gut before absorption.

The most extensive series of observations in which food intake and energy exchange could be correlated were in the oxygen-use studies where the rats lived in the metabolism chambers. Those rats with metabolism increased by the feeding of benzedrine (fig. 1) were eliminated from the present calculations. There remained twenty-two control periods and twenty-one medication periods on seven rats, totaling 89 days and 77 days, respectively. In the non-medication periods, the rats ate an average of 10.16 gm. a day and gained 1.22 gm. daily. During the drug-periods the food intake was decreased to 7.40 gm. per day, and an average of

1.38 gm. weight was lost daily. The benzedrine obviously decreased the food intake in the same way as in the other series described above. Calculation of the weight equivalent of the deficit in food intake accounted for a loss of only 1.21 gm. a day, if pure body fat were being used up. However, the net decrease in body weight was really 2.60 gm. a day, which can be accounted for satisfactorily if the rats were burning also some carbohydrate or protein, or had small shifts in water balance, or both.

Therefore, it appears that the observed changes in body weight in these various series of experiments can be explained by the measured changes in food intake, even when the doses of the drugs administered were so low as not to increase the energy output in spontaneous activity, or the total oxygen use. The effects of benzedrine and propadrine on appetite thus appear to be the primary explanation of the weight reducing power of these drugs.

SUMMARY AND CONCLUSIONS

1. Benzedrine and propadrine cause a loss of body weight in white rats in a manner comparable to that reported for patients.

2. Energy output changes as measured in revolving cages do not explain the decreases in body weight observed. Direct calculation of the energy metabolism from oxygen use in both acute and prolonged experiments showed that, while benzedrine increased the oxygen consumption for about 6 hours after subcutaneous injection, it did not change the total oxygen used in 24 hours unless the doses approached the fatal. Subcutaneous injections of propadrine did not change the metabolism even temporarily.

3. Both benzedrine and propadrine slowed the rate of passage of food through the gastrointestinal tract. This was not a direct local action on the intestine before systemic distribution since the same effects were obtained after subcutaneous injection of smaller doses. The slower passage through

the gut apparently did not modify appreciably the digestion or assimilation of food, since it produced no change in the dry weight of the stools.

4. In paired feeding experiments, medicated rats lost more weight than could be accounted for by decreases in food intake. These rats, however, ingested such a high dosage of the drugs as to bring them into the range where metabolic stimulation probably resulted. There was also the possibility of decreased energy utilization of the unmedicated control rats because of restricted food intake.

5. The food intake was markedly impaired by these drugs, especially during the first few days of medication when the weight changes were greatest. The decreased food intake was sufficient to explain the loss in body weight on the assumption that the energy of the food deficit was being made up mainly by burning body fat, or in some experiments by additional carbohydrate or protein, or by possible concomitant minor shifts in water balance.

6. The main result applicable to the clinical effects in obesity was this diminished food intake. It is noteworthy that a tolerance to the appetite effect of the drugs quickly developed, so that food consumption returned to normal after about a week.

These results indicated that benzedrine and propadrine might be of greatest value in those cases of obesity where control of the appetite was the most pressing need, and where the dosage could be kept low and duration of medication short to minimize the development of tolerance. Propadrine was weaker than benzedrine, but since it lacked the unpleasant central excitant effects of the latter, might well be used first in those patients where this type of treatment is desired. These results, taken as a whole, indicate that benzedrine or propadrine can probably serve only as an adjuvant to the already well recognized and commonly employed measures in controlling obesity.

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STUDIES OF THE COMPARATIVE NUTRITIVE VALUE OF FATS

I. GROWTH RATE AND EFFICIENCY OF CONVERSION OF VARIOUS DIETS TO TISSUE [1]

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FIVE FIGURES

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There are conflicting reports in the literature as to whether various animal and vegetable fats have similar nutritive properties aside from their vitamin content. It is known that with the exception of a few fats having abnormally high melting points, all are equally well absorbed in human subjects, the coefficients of digestibility approaching 95% (Holmes and Deuel, '21; Deuel and Holmes, '22). The requirement for certain unsaturated fatty acids present in a number of vegetable oils has been well established (Burr and Burr, '30). It has also been postulated by Schantz, Elvehjem and Hart ('40) that butter fat possesses specific nutritive properties since they found greater growth of rats during the first 6 weeks following weaning when the diet consisted of a homogenized mixture of skimmed milk and butter fat than

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when cottonseed, corn, coconut or soybean oils were substituted for butter fat. These investigators believe that this stimulatory effect is traceable to some component of the fat itself rather than of the unsaponifiable residue. It could not be ascribed to the phospholipid (Schantz, Boutwell, Elvehjem and Hart, '40a). But the administration of the saturated nonvolatile acids of butter fat brought about the greater growth, while the unsaturated and volatile acids were inactive (Schantz, Boutwell, Elvehjem and Hart, '40b). Boutwell et al. ('41) found that the stimulatory factor could also be produced by hydrogenation of the unsaturated fraction in butter, but not by employing a similar procedure on the unsaturated fraction of the vegetable oils. In their most recent report, Boutwell et al. ('43) have found that the superiority of butter is only evident when the carbohydrate is lactose, while superior growth occurred with corn oil when the diet contained dextrin, sucrose, glucose or starch. Boer ('41) has also postulated that butter fat contains a specific growth-promoting property since he was able to obtain better growth in rats with butter than with olive oil diets. Gullickson, Fountaine and Fitch ('42) have reported that butter fat, tallow and lard are better tolerated by young calves and cause better growth than corn, cottonseed, soybean and coconut oils when the fats are homogenized in skim milk.

However, Euler, Euler and Saberg ('41) obtained superior growth in rats on margarine diets as compared with butter diets over a 6-week period where the average gain on margarine was 96.7 gm. compared with a mean gain on the butter diet of 77 gm. Freeman and Ivy ('42) found no statistically significant differences in early growth of rats fed milk with coconut oil as compared with butter fat although the butter-fed rats were larger after 3 months. These results are diametrically opposed to those of Harris and Mosher ('40) who found more rapid growth of weanling rats on butter fat and of older rats on coconut oil. In view of these apparent contradictions the following experiments were undertaken to follow growth rate, efficiency of conversion of food calories

into body tissue, the comparative composition of the body tissues of such rats, the effect of flavor on food preference and a comparison of pregnancy and lactation on such diets. The latter topics will be dealt with in later reports.

EXPERIMENTAL

Three series of tests were made on a total of 250 male and 309 female rats. In series I, the growth of twenty-two and twenty-four male rats was followed over 6 weeks on the butter and margarine diets described below; also the growth of two groups of female rats consisting of twenty-eight and twenty-four in the first case and eighteen and nineteen in the second set was followed over a 6-week period on the butter and margarine diets respectively. In series II and III, approximately fifteen rats of each sex were used with diets made up of each of the following fats over a 12-week period: a butter fat or a whole butter, corn oil, cottonseed oil, a margarine fat or a whole margarine, olive oil, peanut oil, or soybean oil. In series III whole butter and margarine were used and the diets were flavored by the addition to the fats of 4 parts per million of diacetyl which is the level preferred by the rats (Deuel and Movitt, '44).

The diets employed are listed in table 1.

The diets were prepared weekly and stored in the refrigerator to prevent rancidity. Several days' supply was placed at one time in the food cups. By the use of a special type of hopper practically no spilling of the food occurred and the food consumption could be determined with considerable accuracy. Animals were weighed weekly. Also at 3 and 6 weeks, the left tibia was x-rayed with a McFedries dental x-ray apparatus, the rats being lightly anesthetized with ether during this procedure. The length of the bones was determined by reading the x-ray photographs with a cathetometer.

The animals used were from our stock colonies. The litters were equally distributed between the various diets; when more than two in a litter developed diarrhea during the first week, the whole litter was discarded. To avoid differences in growth

TABLE 1

Composition of diets used.

COMPONENT		DIET I	DIET II
Mineralized skimmed milk powder [1]		70.6	70.6
Added minerals (per 100 lbs.) [2]			
$MnSO_4 \cdot 7H_2O$	3.455 gm.		
$CuSO_4 \cdot 5H_2O$	3.340 gm.		
$Fe \cdot C_6H_8O_7 \cdot 3H_2O$	45.557 gm.		
Butter fat [3] or margarine fat [4]		29.4	
Vitamin D concentrate (per 1000 gm. fat) [5]	801 mg.		
Butter fat,[6] margarine fat [6 7], or vegetable oils supplemented as follows per 1000 gm. fat			29.4
Vitamin A: carotene [8] (40% of total A)	8 mg.		
vitamin A concentrate [9]	133 mg.		
Vitamin D: viosterol [10]	801 mg.		
Vitamin E: a tocopherol [11]	10 mg.		
Diacetyl [12] or commercial butter flavor [13]	4 mg.		

[1] Challenge brand prepared by roller process.

[2] Calculated to give 1.5 mg. Fe and 0.15 mg. each of Cu and Mn per 8 gm. of food.

[3] Challenge brand.

[4] Fortified with 7500 I.U. of vitamin A per pound.

[5] 15,000 U.S.P. XI units per gram. Calculated to furnish 28.2 units of vitamin D per 8 gm. of food.

[6] In series III where whole butter or whole margarine was used, these were increased so that the same amount of fat was present.

[7] Fortified with 9000 I.U. per pound.

[8] S.M.A. 90% beta and 10% alpha carotene. This gives 18.8 μg. or 31.4 I.U. per 8 gm. of food.

[9] Kindly furnished by the Atlantic Coast Fisheries. Potency was 150,000 I.U. per gram or 45 I.U. per 8 gm. of food.

[10] Calculated to give 18.8 U.S.P. XI units per gm. of food.

[11] Calculated to give 23.5 μg. per 8 gm. of food.

[12] Obtained from Larkin and Co., Buffalo, N. Y.

[13] B F A obained from Verley Products Corporation, 1621 Carroll Ave., Chicago, Ill.

ascribable to environmental conditions, the animals on the various diets were so located as to prevent any segregation according to diet.

The butters for the experiments in series II and III were of highest quality obtained in equal amounts from Minnesota, Wisconsin, Texas, Oregon [2] and California. The margarine,

[2] From farm of Oregon State College through courtesy of Dr. J. S. Butts.

in which the fat was a hydrogenated cottonseed oil, was purchased on the open market in New York, Chicago and Los Angeles. These samples were forwarded by express packed in solid carbon dioxide. For series II the butter and margarine were melted, the water and protein allowed to settle and the fat filtered out and mixed to give a homogenous sample. For series III, they were softened sufficiently to be mixed satisfactorily. The oils were obtained directly from the producer and shipped to us by express.[3] They were stored in the refrigerator at approximately 38°F. in air tight containers in the dark, which procedure kept them fresh during the course of the experiments. Inasmuch as the rat is very sensitive to even slight evidences of rancidity in fats, any specimen of fat or oil which gave positive organoleptic evidence of rancidity was not used in this investigation. The free fatty acid content, saponification number, iodine number, and thiocyanogen number of the fats were also determined. These were normal except that the free fatty acid content in the olive oil samples were especially high, being 2.20 and 4.37% in the samples used in series II and III respectively although they possessed excellent flavors.

RESULTS

The growth rate of the male and female rats receiving the butter or margarine diets (diet I) over a 6-week period is illustrated in figure 1.

The growth of the rats on the various diets in series II and series III was similar for the first 6 weeks. Figures 2 and 3 give the average gain of male and female rats respectively on the seven different fats over a 6-week period. Figure 4 gives the average total gain of the rats in series II and series III after 12 weeks on the respective diets.

[3] Cotton and peanut oils from Southern Cotton Oil Co., Savannah, Ga.; corn oil from local distributor, Corn Products Refining Co., soybean oil from Staley Manufacturing Co., Decatur, Ill., and olive oil from Neapolitan Olive Oil Products, Los Angeles.

The average tibia lengths of the rats in series III are given in figure 5 for 3 and 6 weeks on each diet. Similar results were obtained on series II for 6 weeks but no measurements were made in this series at 3 weeks.

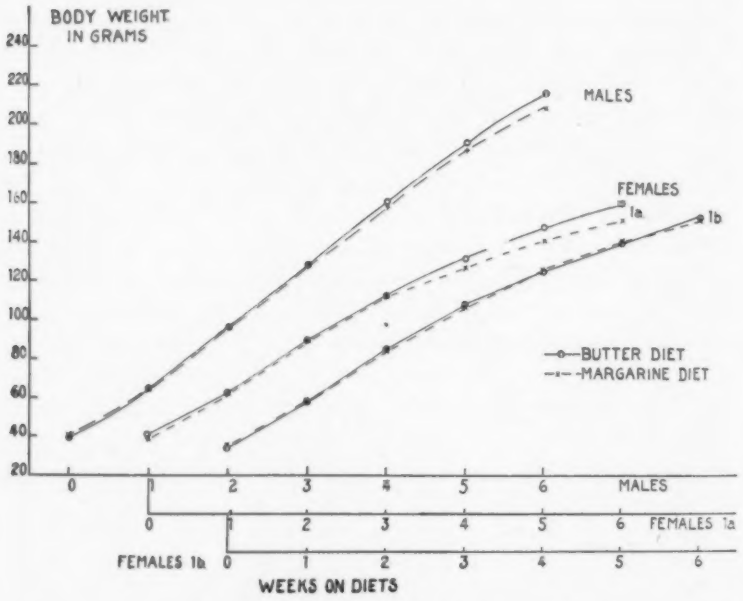

Fig. 1 The rate of growth of male and female rats on diets of mineralized skimmed milk powder mixed with butter fat or margarine fat. The following number of rats were used in the butter and margarine tests respectively: males 22 and 24; females in Ia, 28 and 24, and in Ib, 18 and 19.

The efficiency of transformation of the various diets to body tissue during the first 6 weeks is illustrated in table 2. Because of the slowing down of growth after that period, calculations of these ratios beyond that point are not included. Since the ratio of total food consumed to gain in body weight approaches infinity when the increase in weight approaches zero, we have omitted from the averages all experiments where the growth was 10 gm. or less. Practically no experi-

ments had to be dropped for this reason except with the females on fifth and sixth weeks. However, a clearer picture is probably obtained in the second average where all tests are included. This was obtained by dividing the total food consumed by all rats for the 6-week period by the total gain in weight of these animals over this interval.

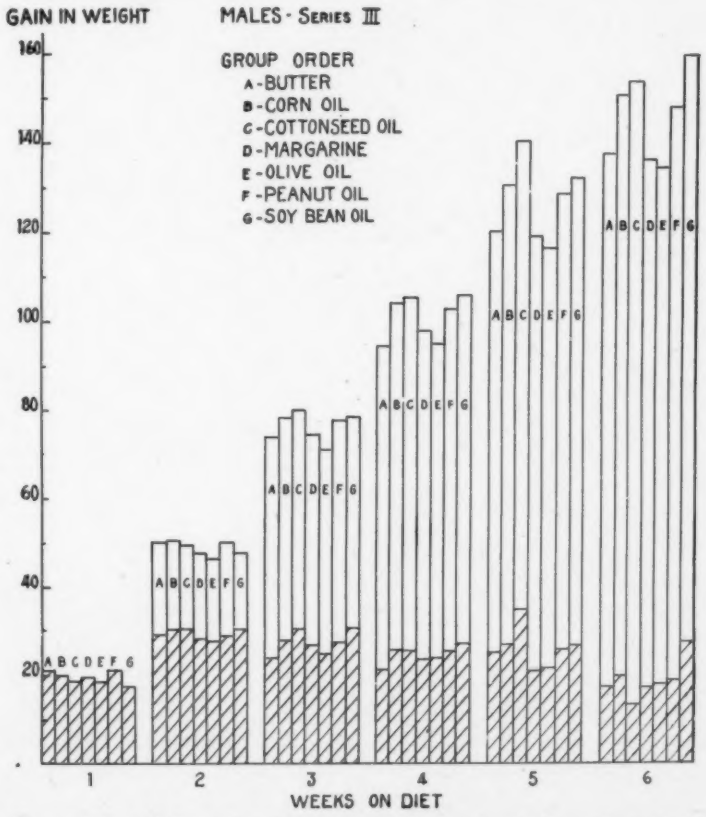

Fig. 2 The shaded portion represents growth of male rats for each week while the unshaded portion represents total growth from initiation of the experiment. The following number of animals were employed: butter (13), corn oil (13), cottonseed oil (14), margarine (13), olive oil (13), peanut oil (13), soybean oil (13).

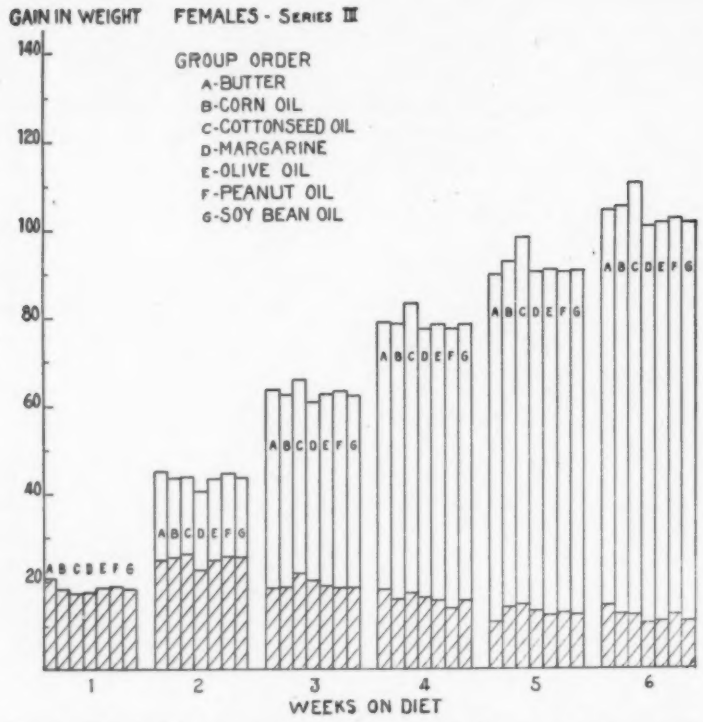

Fig. 3 The shaded portion represents growth of female rats for each week while the unshaded portion represents total growth from initiation of experiments. The following number of animals were employed: butter (15), corn oil (16), cottonseed oil (15), margarine (15), olive oil (13), peanut oil (15), soybean oil (15).

DISCUSSION

No differences were found in the rate of growth when weanling rats were placed for a period of 12 weeks on diets composed of mineralized skimmed milk powder, vitamin supplements and the different fats under investigation. The gains in weight of the male rats in series III at 3, 6 and 12 weeks were as follows: 3 weeks, butter 73.9, corn oil 78.1, cottonseed oil 79.7, margarine 74.3, olive oil 70.8, peanut oil 77.2 and soybean oil 78.4 gm.; 6 weeks, 137.3, 150.2, 153.6, 137.5, 134.0,

147.2 and 159.2 for the groups on the different diets respectively; 12 weeks, 225.5, 230.3, 230.6, 205.0, 206.5, 228.0 and 243.1 gm. With the female rats in the same series, the results were: 3 weeks, 63.8, 62.5, 65.8, 60.8, 62.6, 63.2 and 62.4 gm.;

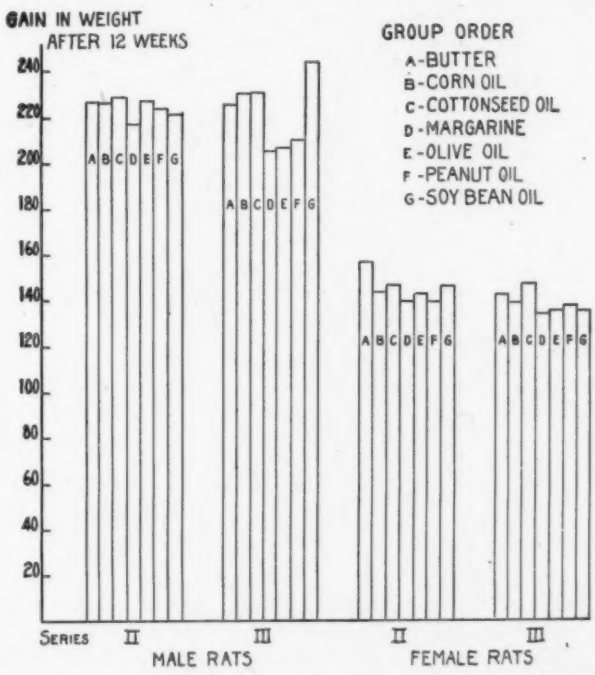

Fig. 4 The total gain of male and female rats in series II and III on the diets containing the various fats over a 12-week period. The following number of rats were employed: male rats (series II and III respectively — butter (12, 13), corn oil (14, 13), cottonseed oil (14, 14), margarine (12, 13), olive oil (16, 13), peanut oil (13, 13), soybean oil (13, 13); female rats — butter (15, 15), corn oil (14, 16), cottonseed oil (15, 15), margarine (18, 15), olive oil (12, 13), peanut oil (17, 15), soybean oil (17, 15).

6 weeks, 104.1, 105.0, 110.2, 100.5, 101.1, 102.3 and 101.3; 12 weeks, 142.4, 137.0, 147.2, 133.7, 134.8, 136.9 and 134.3 gm. In no single case was the average growth of the rats on the butter diet the highest. In the tests on male rats, it was sixth

at 3 and 6 weeks and fifth at 12 weeks. With the females the butter group was either second or third. However, in only a few cases were the differences statistically significant. Based on a greater value than 3.00 being significant for the ratio of mean difference to standard error of mean difference, the

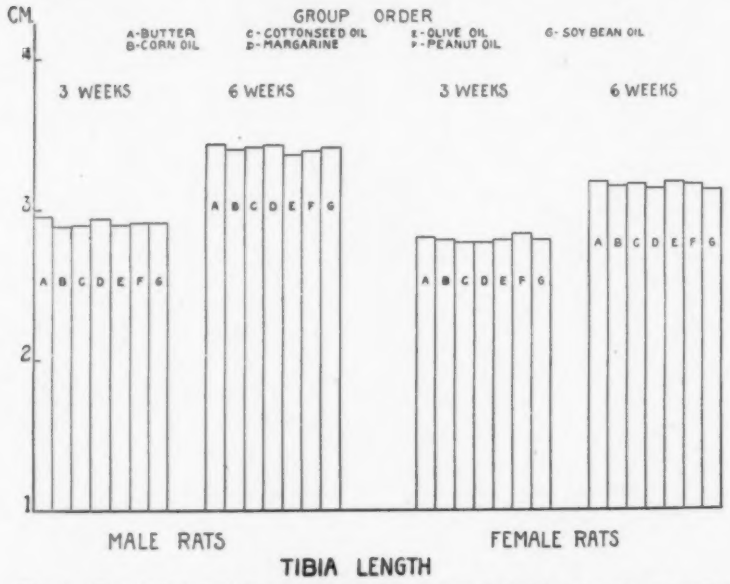

Fig. 5 The tibia length in centimeters for male and female rats after 3 and 6 weeks on various diets. The averages are for the following number of animals: male rats (3 and 6 weeks respectively) — butter (12, 13), corn oil (12, 10), cottonseed oil (13, 12), margarine (14, 12), olive oil (13, 12), peanut oil (13, 12), soybean oil (13, 9); female rats — butter (14, 12), corn oil (16, 13), cottonseed oil (15, 13), margarine (15, 13), olive oil (13, 11), peanut oil (15, 13), soybean oil (15, 13).

weights between the sixth and tenth weeks of the male rats receiving the soybean oil diet are statistically higher than those of the rats receiving butter, margarine or olive oil in the diet. On the other hand, the females on soybean oil did slightly poorer than the rats on the butter diet; however, in the tests with females there is no significant statistical difference between any fats at any period of the study.

TABLE 2

The ratio of food intake to gain in weight.

DIET	1ST WEEK	2ND WEEK	3RD WEEK	4TH WEEK	5TH WEEK	6TH WEEK	MEAN AVERAGE	TOTAL FOOD CONSUMED: TOTAL GAIN IN WEIGHT [1]
Male rats								
Butter [2]	1.44	1.75	2.60	2.86	2.86	3.74	2.54	2.54
	(15)	(15)	(14)	(13)	(15)	(12)	...	...
Corn oil	1.44	1.55	2.23	2.66	2.63	3.65	2.36	2.35
	(15)	(15)	(15)	(15)	(14)	(14)	...	...
Cottonseed oil	1.38	1.51	2.01	2.63	2.63	3.24	2.23	2.22
	(14)	(15)	(15)	(15)	(15)	(13)	...	...
Margarine [2]	1.50	1.71	2.38	2.82	3.16	3.61	2.53	2.38
	(15)	(16)	(16)	(16)	(14)	(13)	...	...
Olive oil	1.36	1.59	2.22	2.60	3.31	2.86	2.33	2.29
	(13)	(15)	(15)	(15)	(15)	(11)	...	...
Peanut oil	1.40	1.69	2.25	2.55	2.67	3.66	2.37	2.33
	(15)	(15)	(15)	(15)	(15)	(14)	...	...
Soybean oil	1.51	1.57	2.02	2.50	2.51	2.96	2.18	2.17
	(13)	(15)	(15)	(14)	(14)	(15)	...	...
Female Rats								
Butter [2]	1.47	1.73	2.95	3.65	4.09	4.30	3.03	2.78
	(14)	(15)	(14)	(14)	(7)	(11)	...	...
Corn oil	1.55	1.86	2.82	3.48	4.36	4.41	3.08	2.95
	(14)	(15)	(14)	(14)	(14)	(10)	...	...
Cottonseed oil	1.54	1.67	2.61	3.31	4.02	4.66	2.97	2.71
	(15)	(15)	(15)	(15)	(14)	(10)	...	...
Margarine [2]	1.67	1.79	2.50	3.23	4.04	4.58	2.97	2.90
	(15)	(13)	(14)	(14)	(10)	(7)	...	...
Olive oil	1.54	1.78	2.43	3.48	3.99	4.56	2.96	2.70
	(14)	(14)	(14)	(11)	(9)	(6)	...	...
Peanut oil	1.63	1.73	2.87	3.40	3.99	4.27	2.98	2.80
	(15)	(15)	(15)	(11)	(11)	(8)	...	...
Soybean oil	1.52	1.69	3.05	3.40	4.16	4.87	3.12	2.95
	(14)	(15)	(14)	(13)	(10)	(7)	...	...

The figures in parentheses represent the number of animals in the average.

[1] Experiments on all rats in these tests. Total food consumption of each group for 6 weeks divided by total gain in weight for the same rats over entire period.

[2] Corrected for water content of diet.

The uniformity in growth for the first 6 weeks on diets containing either butter fat or margarine fat is further emphasized in the results on series I. These data are also confirmed by series II. With male rats the gains at 3 weeks were 80.7 (butter fat), 84.7 (corn oil), 85.8 (cottonseed oil), 75.6 (margarine fat), 79.3 (olive oil), 83.8 (peanut oil), 82.1 (soybean oil) and at 6 weeks 147.6, 156.7, 160.0, 144.4, 152.7, 152.1 and 149.7 gm., respectively. In the tests on female rats in this series the cottonseed oil group was slightly higher at 3 weeks, the increases in weight being 72.8, 69.3, 74.0, 68.5, 71.3, 68.8 and 70.5 gm. respectively; at 6 weeks the butter group led but no differences were significant. The values at this period were 116.9, 108.7, 113.0, 105.0, 110.2, 105.9 and 110.4 gm., respectively. At 12 weeks in this series as shown in figure 4, the results on the males were identical; the levels on the butter group were slightly higher but in no case significantly different than any other groups from a statistical standpoint, in the case of the females.

That the growth of the rats on the various diets represents a real increase in size is demonstrated by the fact that a similar bone growth obtained. The length of the tibia in the various groups was found identical after 3 and 6 weeks on the diets in series III (fig. 5). It is further confirmed by the results at 6 weeks only in series II where the tibia lengths in centimeters were found to be as follows for the seven diets: male, 3.30, 3.36, 3.39, 3.28, 3.37, 3.44, 3.35 and females, 3.14, 3.13, 3.16, 3.06, 3.16, 3.10 and 3.14.

Moreover, further evidence of the similarity in nutritive value of the various fats is obtained from the results of the efficiency of transformation of the food to body tissues (table 2). Identical values were obtained at all periods and there is no case where the discrepancies noted by Schantz et at. ('40) were found.

There are several explanations as to why these results and those of Schantz et al. ('40) are contradictory. We mixed the fats with skimmed milk powder rather than with the liquid skimmed milk, and this renders a more uniform diet available

to the growing rat throughout each day. In their last study, Boutwell et al. ('43) indicate that in their earlier experiments the fat "tended to separate out of the liquid diet so that the rats ate an abnormally high fat diet soon after feeding time, leaving nearly skim milk to eat the rest of the day". Obviously with such irregularities in composition of the milk, one cannot accept as valid the differences in efficiency of transformation of the various skimmed milk diets to body tissue reported by Schantz et al. ('40).

Secondly, in our tests, the food consumption was approximately the same in all groups. Since growth is so closely related to the intake of nutrient materials, it is obvious that decreased food intake per se may be the cause of decreased growth. Boutwell et al. ('41) have indicated that the lower growth rate of animals on the corn oil diet as compared with those on the butter or butter fat fractions was associated with a smaller daily consumption of food. However, they indicate that the increased appetite and greater palatability are the attributes of a superior food. We believe that there are additional factors involved in appetite and food consumption. Although we have found that animals in general prefer a butter diet to one containing the other vegetable fats, they will also overwhelmingly prefer a diet of peanut oil with commercial butter flavor to one which is unflavored. Obviously, it is flavor rather than nutritive value which guides the choice here.

Thirdly, in the original paper of Schantz et al. ('40), there are wide variations in starting weight favorable to the butter group. In the series of male rats the cottonseed and soybean oil groups weighed on an average only 32 gm. compared with 42 gm. for those on butter; with the females the comparative groups weighed 30 and 39 gm., respectively. In our experiments in series I, the ten animals lowest in weight at the start of the tests (32.6 gm.) gained only 103.4 gm. while the ten heaviest weanling rats (42.8 gm.) increased by 130.8 gm. over a 6-week period. In the tests reported in the present paper

the average weights of the various groups varied less than 3 gm. in any series of tests.

Furthermore, when small numbers of rats are used in each group, it is imperative that litter mates be distributed throughout the various groups. In the present work there were three litters where the rats consistently gained far under the average on the various diets. When a small number of animals is used, variations might be noted especially if two from such a litter were assigned to one group. It should also be noted that in the experiments of Schantz et al. ('40), only carotene was used as a supplement for vitamin A in the tests with the vegetable oils while obviously the animals on butter diet received also vitamin A. In the present tests, both the provitamin A and vitamin A have been supplied in all diets.

Boutwell et al. ('43) have extracted the skimmed milk powder extensively in their most recent tests while we have used unextracted milk powder where the lipids amounted to 1.10%. However, in the earliest tests of Schantz et at. ('40) where liquid skimmed milk was used, no such extraction was employed; it was in these experiments where the most marked variations in growth occurred at 3 weeks.

The reason for the poor results of Boer ('41) with olive oil might in all probability be ascribed to the strong flavor. These differences were not observed in our studies. The olive oil used by us was the poorest of the fats, judged by the relatively high free fatty acid content. Also Clausen, Barnes and Burr ('43) have shown that the growth on diets containing various fats is markedly influenced by rancidity; they have indicated that "The inadvertent destruction of dietary essentials and the possible independent toxicity of rancid fat are factors that might confuse the interpretation of many diet experiments".

SUMMARY

No differences in the growth of weanling rats were noted at any time over a 12-week period when they were fed minerized skimmed milk powder, vitamin supplements and butter as compared with corn, cottonseed, olive, peanut or soybean

oils or margarine. The extent of growth was confirmed at 3 and 6 weeks by x-ray determinations of tibia length. Also, the efficiencies of conversion of these various fats to body tissue were identical. These experiments refute the idea that butter fat possesses certain saturated fatty acids, not present in other fats, which are essential for growth.

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